



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 5/10, 7/04, 9/00, C07K 14/00, 14/005, 14/15, 14/54	A2	(11) International Publication Number: WO 95/30763 (43) International Publication Date: 16 November 1995 (16.11.95)		
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> (21) International Application Number: PCT/US95/05789 (22) International Filing Date: 9 May 1995 (09.05.95) (30) Priority Data: 08/240,030 9 May 1994 (09.05.94) US (71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street, San Diego, CA 92121 (US). (72) Inventor: RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US). (74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US). </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> (81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>Without international search report and to be republished upon receipt of that report.</i> </td> </tr> </table>			(21) International Application Number: PCT/US95/05789 (22) International Filing Date: 9 May 1995 (09.05.95) (30) Priority Data: 08/240,030 9 May 1994 (09.05.94) US (71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street, San Diego, CA 92121 (US). (72) Inventor: RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US). (74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).	(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
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(54) Title: RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE <div style="text-align: center; margin: 10px 0;"> A TAT ATA TAT ATC GAT ACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC ▶ Met Gly Gln Thr Val Thr Thr Pro Leu Ser CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC ▶ Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC ▶ Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT ▶ Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT ▶ Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT ▶ Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC ▶ Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC ▶ Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser NorI TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA CCT AAA C ▶ Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ???????? </div>				
(57) Abstract Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral <i>gag/pol</i> or <i>env</i> coding sequences. In addition, <i>gag/pol</i> , and <i>env</i> expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, <i>gag/pol</i> and <i>env</i> expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.				

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Description**RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE**5 Technical Field

The present invention relates generally to retroviral vectors for use in gene transfer, and more specifically, to retroviral vectors which are constructed such that the formation of replication competent virus by recombination is precluded.

10 Background of the Invention

Retroviruses are RNA viruses which can replicate and integrate into a host cell's genome through a DNA intermediate. This DNA intermediate, or provirus, may be stably integrated into the host's cellular DNA. Retroviruses are known to be responsible for a wide variety of diseases in both man and animals, including for example
15 AIDS and a wide variety of cancers.

Although retroviruses can cause disease, they also have a number of properties that lead them to be considered as one of the most promising techniques for genetic therapy of disease. These properties include: (1) efficient entry of genetic material (the vector genome) into cells; (2) an active efficient process of entry into the
20 target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target particular cellular subtypes through control of the vector-target cell binding and tissue-specific control of gene expression. In using a retrovirus for genetic therapy, a foreign gene of interest may be incorporated into the retrovirus in place of normal retroviral RNA. When the retrovirus injects its
25 RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of foreign protein by the host cell.

Most retroviral vector systems which have been developed for gene therapy are based on murine retroviruses. Briefly, these retroviruses exist in two forms,
30 as proviruses integrated into a host's cellular DNA, or as free virions. The virion form of the virus contains the structural and enzymatic proteins of the retrovirus (including reverse transcriptase), two RNA copies of the viral genome, and portions of the cell's plasma membrane in which is embedded the viral envelope glycoprotein. The genome is organized into four main regions: the Long Terminal Repeat (LTR), and the *gag*, *pol*,
35 and *env* genes. The LTR may be found at both ends of the proviral genome, is a composite of the 5' and 3' ends of the RNA genome, and contains *cis*-acting elements

necessary for the initiation and termination of transcription. The three genes *gag*, *pol*, and *env* are located between the terminal LTRs. The *gag* and *pol* genes encode, respectively, internal viral structures and enzymatic proteins (such as integrase). The *env* gene encodes the envelope glycoprotein (designated gp70 and p15e) which confers infectivity and host range specificity of the virus, as well as the "R" peptide of undetermined function.

An important consideration in using retroviruses for gene therapy is the availability of "safe" retroviruses. Packaging cell lines and vector producing cell lines have been developed to meet this concern. Briefly, this methodology employs the use of two components, a retroviral vector and a packaging cell line (PCL). The retroviral vector contains long terminal repeats (LTRs), the foreign DNA to be transferred and a packaging sequence (ψ). This retroviral vector will not reproduce by itself because the genes which encode structural and envelope proteins are not included within the vector genome. The PCL contains genes encoding the *gag*, *pol*, and *env* proteins, but does not contain the packaging signal " ψ ". Thus, a PCL can only form empty virion particles by itself. Within this general method, the retroviral vector is introduced into the PCL, thereby creating a vector-producing cell line (VCL). This VCL manufactures virion particles containing only the retroviral vector's (foreign) genome, and therefore has previously been considered to be a safe retrovirus vector for therapeutic use.

There are, however, several shortcomings with the current use of VCLs. One issue involves the generation of "live virus" (*i.e.*, replication competent retrovirus; RCR) by the VCL. Briefly, RCR can be produced in conventional producer cells when: (1) The vector genome and the helper genomes recombine with each other; (2) The vector genome or helper genome recombines with homologous cryptic endogenous retroviral elements in the producer cell; or (3) Cryptic endogenous retroviral elements reactivate (*e.g.*, xenotropic retroviruses in mouse cells).

Another issue is the propensity of mouse based VCLs to package endogenous retrovirus-like elements (which can contain oncogenic gene sequences) at efficiencies close to that with which they package the desired retroviral vector. Such elements, because of their retrovirus-like structure, are transmitted to the target cell to be treated at frequencies that parallel its transfer of the desired retroviral vector sequence.

A third issue is the ability to make sufficient retroviral vector particles at a suitable concentration to: (1) treat a large number of cells (*e.g.*, 10^8 - 10^{10}); and (2) manufacture vector particles at a commercially viable cost.

In order to construct safer PCLs, researchers have generated deletions of the 5' LTR and portions of the 3' LTR of helper elements (*see*, Miller and Buttimore, *Mol. Cell. Biol.* 6:2895-2902, 1986). When such cells are used, two recombination events are necessary to form the wild-type, replication competent genome. Nevertheless, results from several laboratories have indicated that even when several deletions are present, RCR may still be generated (*see*, Bosselman et al., *Mol. Cell. Biol.* 7:1797-1806, 1987; Danos and Mulligan, *Proc. Nat'l. Acad. Sci. USA* 81:6460-6464, 1988). In addition, cell lines containing both 5' and 3' LTR deletions which have been constructed have thus far not proven useful since they produce relatively low titers (Dougherty et al., *J. Virol.* 63:3209-3212, 1989).

One of the more recent approaches to constructing safer packaging cell lines involves the use of complementary portions of helper virus elements, divided among two separate plasmids, one containing *gag* and *pol*, and the other containing *env* (*see*, Markowitz et al., *J. Virol.* 62:1120-1124; and Markowitz et al., *Virology* 167:600-606, 1988). One benefit of this double-plasmid system is that three recombination events are required to generate a replication competent genome. Nevertheless, these double-plasmid vectors have also suffered from the drawback of including portions of the retroviral LTRs, and therefore remain capable of producing infectious virus.

The present invention overcomes the difficulties of recombination and low titer associated with many of the prior packaging cell lines, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions and methods for the construction of packaging cell lines which preclude the formation of RCR by homologous recombination. Within one aspect of the invention, recombinant retroviral vector constructs (RETROVECTOR™) are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis, and a 3' LTR, wherein the retroviral vector construct lacks *gag/pol* and *env* coding sequences. Within one embodiment of the invention, the retroviral vector construct lacks an extended packaging signal. Within one embodiment, the retroviral vector construct lacks a retroviral nucleic acid sequence upstream of the 5' LTR. Within a preferred embodiment, the retroviral vector constructs lack an *env* coding sequence upstream of the 5' LTR. Retroviral vector constructs of the present invention may be constructed from one or more retroviruses, including, for example, a

wide variety of amphotropic, ecotropic, xenotropic, and polytropic viruses (*see e.g.*, Figures 17A, B, and C).

As noted above, retroviral vector constructs of the present invention include one or more heterologous sequences. Within certain embodiments of the invention, the heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7 and 8. Within one embodiment, the heterologous sequence is a gene encoding a cytotoxic protein, such as, for example, ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Within other embodiments the heterologous sequence may be an antisense sequence, or an immune accessory molecule. Representative examples of immune accessory molecules include IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, and IL-14. Particularly preferred immune accessory molecules may be selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon, or the group consisting of ICAM-1, ICAM-2, β -microglobulin, LFA3, HLA class I and HLA class II molecules.

Within other embodiments of the invention, the heterologous sequence may encode a gene product that activates a compound with little or no cytotoxicity into a toxic product. Representative examples of such gene products include type I thymidine kinases such as HSVTK and VZVTK. Within another embodiment, the heterologous sequence may be a ribozyme. Within yet other embodiments, the heterologous sequence is a replacement gene, which encode proteins such as Factor VIII, ADA, HPRT, CF and the LDL Receptor. Within other embodiments, the heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

Within other aspects of the present invention, *gag/pol* expression cassettes are provided, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within one embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other aspects *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the expression cassette does not co-encapsidate with a replication competent virus.

Within another aspect of the present invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *gag/pol* gene has been deleted without effecting the biological activity of integrase. Within one embodiment, a

5' terminal end of the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within a further embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other embodiments, the 3' terminal end has been deleted upstream (5') of nucleotide 5751 of SEQ ID NO: 1.

5 Within other aspects of the present invention, *env* expression cassettes are provided, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 retroviral nucleotides are included upstream of the *env* gene. Within another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein the
10 *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene. Within yet another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted without effecting the biological activity of env. Within one embodiment, the 3'
15 terminal end of the gene has been deleted such that a complete R peptide is not produced by the expression cassette. Within a further embodiment, the *env* gene is derived from a type C retrovirus, and the 3' terminal end has been deleted such that the *env* gene includes less than 18 nucleic acids which encode the R peptide. Within a preferred embodiment, the 3' terminal end has been deleted downstream from nucleotide 7748 of
20 SEQ ID NO: 1.

 Within various embodiments of the invention, the promoters of the *gag/pol* and *env* expression cassettes described above are heterologous promoters, such as CMV IE, the HVTk promoter, RSV promoter, Adenovirus major-late promoter and the SV40 promoter. Within other embodiments, the polyadenylation sequence is a
25 heterologous polyadenylation sequence, such as the SV40 late poly A Signal and the SV40 early poly A Signal.

 Within another aspect of the present invention, packaging cell lines are provided, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein the *gag/pol* expression cassette lacks a consecutive sequence of greater than 20,
30 preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 consecutive nucleotides which are found in the *env* expression cassette. Within other aspects, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a
35 consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 nucleotides in common.

Representative examples of such retroviral vector constructs, *gag/pol* and *env* expression cassettes are described in more detail below.

Within yet another aspect of the present invention, producer cell lines are provided comprising a packaging cell line as described above, and a retroviral vector construct. Within another aspect of the present invention, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than eight nucleotides in common.

Within other aspects of the invention, methods of producing a packaging cell line are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette as described above into an animal cell; (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into said selected cell, and (d) selecting a cell which expresses high levels of *env* and thereby producing the packaging cell. Within other aspects of the invention, the *env* expression cassette may be introduced into the cell first, followed by the *gag/pol* expression cassette. Within other aspects, methods are provided for producing recombinant retroviral particles comprising the step of introducing a retroviral vector construct into a packaging cell as described above. Within preferred embodiments, the retroviral vector construct is one of the retroviral vector constructs described above.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

Brief Description of the Drawings

- Figure 1 is a schematic illustration of pKS2+Eco57I-LTR(+).
Figure 2 is a schematic illustration of pKS2+Eco57I-LTR(-).
Figure 3 is a schematic illustration of pKS2+LTR-EcoRI.
Figure 4 is a schematic illustration of pR1.
Figure 5 is a schematic illustration of pR2.
Figure 6 is a schematic illustration of pKT1.
Figure 7 is a schematic illustration of pRI-HIVenv.
Figure 8 is a schematic illustration of pR2-HIVenv.

Figure 9 is a representative "prewobble" sequence for a MoMLV *gag/pol* (see also SEQ I.D. Nos. 11 and 12).

Figure 10 is a representative "wobble" sequence for a MoMLV *gag/pol* (see also SEQ. I.D. Nos. 9 and 10).

5 Figure 11 is a schematic illustration of pHCMV-PA.

Figure 12 is a schematic illustration of pCMV *gag/pol*.

Figure 13 is a schematic illustration of pCMVgpSma.

Figure 14 is a schematic illustration of pCMVgp-X.

Figure 15 is a schematic illustration of pCMV env-X.

10 Figure 16 is a schematic illustration of pRgpNeo.

Figures 17A, B and C comprise a table which sets forth a variety of retroviruses which may be utilized to construct the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention.

Figure 18 is a schematic illustration of pCMV Envam-Eag-X-less.

15 Figure 19A is a diagrammatic illustration of a "wobble" -*gag* construct.

Figure 19B is a diagrammatic illustration of a "normal" -*gag* construct.

Detailed Description of the Invention

20 Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Briefly, the retroviral vector construct must include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (*e.g.*, cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement gene), or which are useful as a molecule itself (*e.g.*, as a ribozyme or antisense sequence). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence, which is of a size sufficient to allow production of viral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 kB in length.

35 The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally,

the retroviral vector construct may also include selectable markers such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more specific restriction sites and a translation termination sequence.

"Expression cassette" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within preferred embodiments of the invention, both the promoter and the polyadenylation sequence are from a source which is heterologous to the helper elements (*i.e.*, *gag/pol* and *env*). Expression cassettes of the present invention may be utilized to express a *gag/pol* gene or an *env* gene. In addition, the expression cassettes may also be utilized to express one or more heterologous sequences either from a *gag/pol* and/or *env* expression cassette, or from an entirely different expression cassette.

Within preferred embodiments of the invention, the expression cassettes described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

PREPARATION OF RETROVIRAL VECTOR CONSTRUCTS, GAG/POL EXPRESSION CASSETTES AND ENV EXPRESSION CASSETTES

As noted above, the present invention provides compositions and methods for constructing packaging cells which preclude the formation of replication competent virus by homologous recombination. The following sections describe the preparation of retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes.

1. Construction of retroviral vector constructs

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological

activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTR's may be readily identified in the provirus due to their precise duplication at either end of the genome.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, tRNA binds to a retroviral tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to 5' and 3' LTRs, a tRNA binding site, and an origin of second strand DNA synthesis, retroviral vector constructs of the present invention also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Retroviral vector constructs of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those set forth below in Figures 17A, B and C (*see* RNA Tumor Viruses at pages 2-7), as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB₉₋₁ (*see* O'Neill et al., *J. Vir.* 53:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly et al., *J. Vir.* 45(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral vector constructs of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus, Gibbon Ape Leukemia Virus, Mason Pfizer Monkey Virus,

and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol.* 19:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral vector constructs, packaging cells, or producer cells of the present invention given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). Further, within certain embodiments of the invention, portions of the retroviral vector construct may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus. Similarly, portions of a packaging cell line may be derived from different viruses (*e.g.*, a *gag/pol* expression cassette may be constructed from a Moloney Murine Leukemia Virus, and an *env* expression cassette from a Mason Pfizer Monkey virus).

As noted above, within various aspects of the present invention, retroviral vector constructs are provided which have packaging signals, and which lack both *gag/pol* and *env* coding sequences. As utilized within the context of the present invention, a packaging signal should be understood to refer to that sequence of nucleotides which is not required for synthesis, processing or translation of viral RNA or assembly of virions, but which is required in *cis* for encapsidation of genomic RNA (*see* Mann et al., *Cell* 33:153-159, 1983; RNA Tumor Viruses, Second Edition, *supra*).

Further, as utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to refer to retrovectors which contain less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retroviral vector construct. Representative examples of such retroviral vector constructs are set forth in more detail below and in Example 1.

As an illustration, within one embodiment of the invention construction of retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing retroviral vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of
5 nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence (counting from the 5' LTR cap site) from approximately nucleotide 144 of SEQ. I.D. No. 1, up through the *Pst* I site (nucleotide 567 of SEQ. I.D. No. 1). The
10 extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1040. Thus, within this embodiment retroviral vector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal downstream of nucleotide 567.

15 Within other embodiments of the invention, retroviral vector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated downstream of the start of the *gag/pol* gene (nucleotide 621 of SEQ ID NO: 1). Within preferred embodiments of the
20 invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615 or 617 of SEQ ID NO: 1.

Within other aspects of the invention, retroviral vector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene (e.g., for MoMLV, beyond nucleotide 621 of SEQ ID NO: 1). When such
25 retroviral vector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*. Such *gag/pol* expression cassettes are described in more detail below in section 2, and in Example 3.

30 Within other aspects of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized
35 within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the retrovector plasmid construct contains less than 20, preferably less than 15, more

preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retroviral vector construct, upstream of and/or contiguous with the 5' LTR. Within preferred embodiments, the retrovector plasmid constructs do not contain an *env* coding sequence (as discussed below) upstream of the 5' LTR. A particularly preferred embodiment of such retrovector plasmid constructs is set forth in more detail below in Example 1.

Within a further aspect of the present invention, retrovector plasmid constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome. A representative example of such a retroviral vector construct is set forth in more detail below in Example 1.

2. Construction of gag/pol expression cassettes

As noted above, the present invention also provides a variety of *gag/pol* expression cassettes which, in combination with the retroviral vector constructs and *env* expression cassettes of the present invention, enable the construction of packaging cell lines and producer cell lines which preclude the formation of replication competent virus. Briefly, retroviral *gag/pol* genes contain a *gag* region which encodes a variety of structural proteins that make up the core matrix and nucleocapsid, and a *pol* region which contains genes which encode (1) a protease for the processing of *gag/pol* and *env* proteins, (2) a reverse transcriptase polymerase, (3) an RNase H, and (4) an integrase, which is necessary for integration of the retroviral provector into the host genome. Although retroviral *gag/pol* genes may be utilized to construct the *gag/pol* expression cassettes of the present invention, a variety of other non-retroviral (and non-viral) genes may also be utilized to construct the *gag/pol* expression cassette. For example, a gene which encodes retroviral RNase H may be replaced with genes which encode bacterial (e.g., *E. coli* or *Thermus thermophilus*) RNase H. Similarly, a retroviral integrase gene may be replaced by other genes with similar function (e.g., yeast retrotransposon TY3 integrase).

Within one aspect of the invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain

codons which are degenerate for gag. Briefly, as noted above, in wild-type retrovirus the extended packaging signal of the retrovirus overlaps with sequences which encode gag and pol. Thus, in order to eliminate the potential of crossover between the retroviral vector construct and the *gag/pol* expression cassette, as well as to eliminate the possibility of co-encapsidation of the *gag/pol* expression cassette and replication competent virus or retroviral vector constructs, sequences of overlap should be eliminated. Within one embodiment of the invention, elimination of such overlap is accomplished by modifying the *gag/pol* gene (and more specifically, regions which overlap with the retroviral vector construct, such as the extended packaging signal) to contain codons that are degenerate (*i.e.*, that "wobble") for gag. In particular, within preferred embodiments of the invention codons are selected which encode biologically active gag/pol protein (*i.e.*, capable of producing a competent retroviral particle, in combination with an *env* expressing element, and a RNA genome), and which lack any packaging signal sequence, including in particular, extended packaging signal sequence. As utilized herein, the phrase "lacks any retroviral packaging signal sequence" should be understood to mean that the *gag/pol* expression cassette contains less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are identical to a sequence found in a retroviral packaging signal (*e.g.*, in the case of MoMLV, extending up and through the *Xho* I site at approximately nucleotide number 1561). A particularly preferred example of such modified codons which are degenerate for gag is shown in Figure 10, and in Example 3, although the present invention should not be so limited. In particular, within other embodiments, at least 25, 50, 75, 100, 125 or 135 *gag* codons are modified or "wobbled" from the native *gag* sequence within the *gag/pol* expression cassettes of the present invention.

In addition to eliminating overlap between the retroviral vector construct and the *gag/pol* gene, it is also preferable to eliminate any potential overlap between the *gag/pol* gene and the *env* gene in order to prohibit the possibility of homologous recombination. This may be accomplished in at least two principal ways: (1) by deleting a portion of the *gag/pol* gene which encodes the integrase protein, and in particular, that portion of the gene which encodes the integrase protein which overlaps with the *env* coding sequence, or (2) by selecting codons which are degenerate for integrase and/or *env*.

Thus, within one aspect of the present invention *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence or signal, wherein a 3' terminal end of the gene has been deleted without effecting the biological activity of the integrase. (The biological activity

of integrase may be readily determined by detection of an integration event, either by DNA analysis or by expression of a transduced gene; *see* Roth et al., *J. Vir.* 65(4):2141-2145, 1991.) As an example, in the Murine Leukemia Virus MoMLV (SEQ ID. NO. 1), the *gag/pol* gene is encoded by nucleotides 621 through 5834. Within this
5 sequence, the protein integrase is encoded by nucleotides 4610 through nucleotide 5834. A portion of the *gag/pol* sequence which encodes integrase also encodes *env* (which begins at nucleotide 5776). Thus, within one embodiment of the invention, the 3' terminal end of the *gag/pol* gene is deleted or truncated in order to prevent crossover with the *env* gene, without effecting the biological activity of the integrase. Within other
10 preferred embodiments, the *gag/pol* gene is deleted at any nucleotide downstream (3') from the beginning of the integrase coding sequence, and preferably prior to the start of the *env* gene sequence. Within one embodiment, the sequence encoding *gag/pol* is a MoMLV sequence, and the *gag/pol* gene is deleted at any nucleotide between nucleotides 4610 and 5576 (of SEQ. I.D. No. 1), including for example, at nucleotides
15 5775, 5770, 5765, 5760, 5755, 5750.

Within other embodiments of the invention, the *gag/pol* expression cassette contains sequences encoding *gag/pol* (and including integrase), while lacking any sequence found in an *env* gene. The phrase "lacking any sequence found in an *env* gene" should be understood to mean that the *gag/pol* expression cassette does not
20 contain at least 20, preferably at least 15, more preferably at least 10, and most preferably less than 8 consecutive nucleotides which are identical to an *env* sequence, and preferably which are found in an *env* expression cassette which will be utilized along with the *gag/pol* expression cassette to form a packaging cell. Such expression cassettes may be readily prepared by selecting codons which are degenerate for integrase, and
25 which do not encode biologically active *env*. (*See Morgenstern and Land, Nuc. Acids Res.* 18:3587-3596, 1990.)

Within other embodiments of the invention, the *gag/pol* expression cassette contains a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences
30 refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the Cytomegalovirus Immediate Early ("CMV IE") promoter, the Herpes Simplex Virus Thymidine Kinase ("HSVTK") promoter, the Rous Sarcoma Virus ("RSV") promoter,
35 the Adenovirus major-late promoter and the SV 40 promoter. Representative examples

of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

Within preferred aspects of the present invention, *gag/pol* expression cassettes such as those described above will not co-encapsidate along with a replication competent virus. One representative method for determination of co-encapsidation is set forth below in Example 8.

3. Construction of env expression cassettes

Within other aspects of the present invention, *env* expression cassettes are provided which, in combination with the *gag/pol* expression cassettes and retroviral vector constructs described above, preclude formation of replication competent virus by homologous recombination, as well as to confer a particular specificity of the resultant vector particle (e.g., amphotropic, ecotropic, xenotropic or polytropic; see Figure 17, as well as the discussion above). Briefly, in a wild-type retrovirus the *env* gene encodes two principal proteins, the surface glycoprotein "SU" and the transmembrane protein "TM", which are translated as a polyprotein, and subsequently separated by proteolytic cleavage. Representative examples of the SU and TM proteins are the gp120 protein and gp41 protein in HIV, and the gp70 protein and p15e protein in MoMLV. In some retroviruses, a third protein designated the "R" peptide" of undetermined function, is also expressed from the *env* gene and separated from the polyprotein by proteolytic cleavage. In the Murine Leukemia Virus MoMLV, the R peptide is designated "p2".

A wide variety of *env* expression cassettes may be constructed given the disclosure provided herein, and utilized within the present invention to preclude homologous recombination. Within one aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein no more than 6, 8, 10, 15, or 20 consecutive retroviral nucleotides are included upstream (5') of and/or contiguous with said *env* gene. Within other aspects of the invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein the *env* expression cassette does not contain a consecutive sequence of greater than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 or 6 consecutive nucleotides which are found in a *gag/pol* gene, and in particular, in a *gag/pol* expression cassette that will be utilized along with the *env* expression cassette to create a packaging cell line.

Within another aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted

without effecting the biological activity of *env*. As utilized herein, the phrase "biological activity of *env*" refers to the ability of envelop protein to be expressed on the surface of a virus or vector particle, and to allow for a successful infection of a host cell. One practical method for assessing biological activity is to transiently transfect the *env* expression cassette into a cell containing a previously determined functional *gag/pol* expression cassette, and a retroviral vector construct which expresses a selectable marker. A biologically functional *env* expression cassette will allow vector particles produced in that transfected cell, to transmit the selectable marker to a naive sensitive cell such that it becomes resistant to the marker drug selection. Within a preferred embodiment of the invention, the 3' terminal end of the *env* gene is deleted or truncated such that a complete R peptide is not produced by the expression cassette. In the representative example of MoMLV, sequence encoding the R peptide (which begins at nucleotide 7734) is deleted, truncated, or, for example, terminated by insertion of a stop codon at nucleotide 7740, 7745, 7747, 7750, 7755, 7760, 7765, 7770, 7775, 7780, or any nucleotide in between.

Within another aspect of the present invention, *env* expression cassettes are provided which contain a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the CMV IE promoter, the HSVTK promoter, the RSV promoter, the Adenovirus major-late promoter and the SV 40 promoters. Representative examples of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

HETEROLOGOUS SEQUENCES

As noted above, the retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes of the present invention may contain (and express) one or more heterologous sequences. A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, cytotoxic genes, antisense sequences, sequences which encode gene products that activate a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, sequences which encode immunogenic portions of disease-associated antigens and sequences which encode immune accessory molecules. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur.*

J. Biochem. 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen, et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez & Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.* 21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987).

Within further embodiments of the invention, antisense RNA may be utilized as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Within other embodiments of the invention, antisense sequences are provided which inhibit, for example, tumor cell growth, viral replication, or a genetic disease by preventing the cellular synthesis of critical proteins needed for cell growth. Examples of such antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein, et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

Within other aspects of the invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes are provided which direct the expression of a gene product that activates a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product. Representative examples of such gene products include varicella zoster virus thymidine kinase (VZVTK), herpes simplex virus thymidine

kinase (HSVTK) (Field et al., *J. Gen. Virol.* 49:115-124, 1980; Munir et al., *Protein Engineering* 7(1):83-89, 1994; Black and Loeb, *Biochem* 32(43):11618-11626, 1993), and *E. coli*. guanine phosphoribosyl transferase (see U.S. Patent Application Serial No. 08/155,944, entitled "Compositions and Methods for Utilizing Conditionally Lethal
5 Genes," filed November 18, 1993; see also WO 93/10218 entitled "Vectors Including Foreign Genes and Negative Selection Markers", WO 93/01281 entitled "Cytosine Deaminase Negative Selection System for Gene Transfer Techniques and Therapies", WO 93/08843 entitled "Trapped Cells and Use Thereof as a Drug", WO 93/08844 entitled "Transformant Cells for the Prophylaxis or Treatment of Diseases Caused by
10 Viruses, Particularly Pathogenic Retroviruses", and WO 90/07936 entitled "Recombinant Therapies for Infection and Hyperproliferative Disorders.") Within preferred embodiments of the invention, the retroviral vector constructs direct the expression of a gene product that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby affecting localized therapy
15 to the pathogenic agent (see WO 94/13304).

Within one embodiment of the invention, retroviral vector constructs are provided which direct the expression of a HSVTK gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in
20 human cells infected with HIV and carrying the vector construct causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAU, FIAC, DHPG). Such drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir and FIAU triphosphates
25 inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA* 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within further aspects of the present invention, retroviral vector
30 constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention may also direct the expression of one or more sequences which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the
35 disease-state in general but are not required or essential for rendering the cell diseased. In addition, antigens are considered to be "immunogenic" if they are capable, under

appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (see WO 93/10814). Representative examples of altered cellular components which are normally associated with tumor cells include ras* (wherein "*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53*, Rb*, altered protein encoded by Wilms' tumor gene, ubiquitin*, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, Platelet Derived Growth Factor ("PDGF") receptor, insulin receptor, Epidermal Growth Factor ("EGF") receptor, and the Colony Stimulating Factor ("CSF") receptor.

"Disease-associated" antigens should also be understood to include all or portions of various eukaryotic, prokaryotic or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV"; see WO 93/15207), Human Papilloma Virus ("HPV"; see WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV"; see EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV"; see WO 93/09070; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV"; U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV"; see WO 93/02805).

Within other aspects of the present invention, the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes described above may also direct the expression of one or more immune accessory molecules. As utilized herein, the phrase "immune accessory molecules" refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include α interferon, β interferon, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun.* 46:3074, 1991; Gubler et al., *PNAS* 88:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-14, IL-15, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics* 26:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature* 331:624-627, 1988), ICAM-2 (Singer, *Science* 255: 1671, 1992), β -microglobulin (Parnes et al.,

PNAS 78:2253-2257, 1981), LFA-1 (Altmann et al., *Nature* 338: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med.* 166(4):923-932, 1987), HLA Class I, HLA Class II molecules, B7 (Freeman et al., *J. Immun.* 143:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

- 5 Within preferred aspects of the present invention, the retroviral vector constructs described herein may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter, or preferably, by additional secondary promoters (e.g., Internal Ribosome Binding Sites or "IRBS"). Within preferred embodiments of the invention, retroviral vector constructs
- 10 direct the expression of heterologous sequences which act synergistically. For example, within one embodiment retroviral vector constructs are provided which direct the expression of a molecule such as IL-15, IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T_H1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments,
- 15 immune presentation and processing of the disease-associated antigen will be increased due to the presence of the immune accessory molecule.

- Within other aspects of the invention, retroviral vector constructs are provided which direct the expression of one or more heterologous sequences which encode "replacement" genes. As utilized herein, it should be understood that the term
- 20 "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect. Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by
- 25 such defects include Cystic Fibrosis ("CF"; see Dorin et al., *Nature* 326:614,), Parkinson's Disease, Adenosine Deaminase deficiency ("ADA"; Hahma et al., *J. Bact.* 173:3663-3672, 1991), β -globin disorders, Hemophilia A & B (Factor VIII-deficiencies; see Wood et al., *Nature* 312:330, 1984), Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; see Jolly et al., *PNAS*
- 30 80:477-481, 1983) and Familial Hypercholesterolemia (LDL Receptor mutations; see Yamamoto et al., *Cell* 39:27-38, 1984).

- Sequences which encode the above-described heterologous genes may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository
- 35 such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England).

Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

Alternatively, known cDNA sequences which encode cytotoxic genes or other heterologous genes may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. *See also* PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

PREPARATION OF RETROVIRAL PACKAGING CELL LINES, AND GENERATION OF RECOMBINANT VIRAL PARTICLES

As noted above, the *gag/pol* expression cassettes and *env* expression cassettes of the present invention may be used to generate transduction competent retroviral vector particles by introducing them into an appropriate parent cell line in

order to create a packaging cell line, followed by introduction of a retroviral vector construct, in order to create a producer cell line (*see* WO 92/05266). Such packaging cell lines, upon introduction of an N2-type vector construct (Armentano et al., *J. of Vir.* 61(5):1647-1650, 1987) produce a titer of greater than 10^5 cfu/ml, and preferably greater than 10-fold, 20-fold, 50-fold, or 100-fold higher titer than similar transduced PA317 cells (Miller and Buttimore, *Mol. and Cell. Biol.* 6(8):2895-2902, 1986).

Within one aspect of the present invention, methods for creating packaging cell lines are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette according into an animal cell, (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into the selected cell, and (d) selecting a cell which expresses high levels of *env*, and thereby creating the packaging cell. Within other aspects of the present invention, methods for creating packaging cell lines are provided comprising the steps of (a) introducing an *env* expression cassette into an animal cell (b) selecting a cell which expresses high levels of *env*, (c) introducing a *gag/pol* expression cassette into the selected cell, and (d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby creating the packaging cell. As utilized herein, it should be understood that "high" levels of *gag/pol* or *env* refers to packaging cells which produce at least *z* times greater *gag/pol* or *env* protein than PA317 cells, wherein *z* is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including for example human, macaque, dog, rat and mouse cells. Particularly preferred cell lines for use in the preparation of packaging cell lines of the present invention are those that lack genomic sequences which are homologous to the retroviral vector construct, *gag/pol* expression cassette and *env* expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (*see Martin et al., PNAS* 78:4892-4896, 1981; *see also* WO 92/05266).

Expression cassettes of the present invention may be introduced into cells by numerous techniques, including for example, transfection by various physical methods, such as electroporation, DEAE dextran, lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991), liposomes of several types (*see e.g., Wang et al., PNAS* 84:7851-7855, 1987); CaPO_4 (Dubensky et al., *PNAS* 81:7529-7533, 1984), DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989), administration of nucleic acids alone (WO

90/11092), or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3: 147-154, 1992).

5 Producer cell lines (also called vector-producing lines or "VCLs") may then be readily prepared by introducing a retroviral vector construct as described above, into a packaging cell line. Within preferred embodiments of the invention, producer cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably 15, more preferably 10, and most preferably 10 or 8
10 nucleotides in common.

PHARMACEUTICAL COMPOSITIONS

Within another aspect of the invention, pharmaceutical compositions are provided, comprising a recombinant viral particle as described above, in combination
15 with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (e.g., lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or oral, nasal, vaginal, sub-lingual, inhalant or rectal administration.

20 Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or
25 proteins such as human serum albumin. A particularly preferred composition comprises a retroviral vector construct or recombinant viral particle in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including
30 water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for
35 breast or other epithelial carcinomas.

Particularly preferred methods and compositions for preserving recombinant viruses are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses" (see WO 94/11414).

5 METHODS OF ADMINISTRATION

Within other aspects of the present invention, methods are provided for inhibiting or destroying pathogenic agents in a warm-blooded animal, comprising administering to a warm-blooded animal a recombinant viral particle as described above, such that the pathogenic agent is inhibited or destroyed. Within various embodiments of
10 the invention, recombinant viral particles may be administered *in vivo*, or *ex vivo*. Representative routes for *in vivo* administration include intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or even directly into a tumor.

Alternatively, the cytotoxic genes, antisense sequences, gene products,
15 retroviral vector constructs or viral particles of the present invention may also be administered to a warm-blooded animal by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS*
20 88:2726-2730, 1991); liposomes of several types (see e.g., Wang et al., *PNAS* 84:7851-7855, 1987); CaPO_4 (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al, *J. of Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3: 147-154, 1992).

25 Within a preferred aspect of the present invention, retroviral particles (or retroviral vector constructs alone) may be utilized in order to directly treat pathogenic agents such as a tumor. Within preferred embodiments, the retroviral particles or retroviral vector constructs described above may be directly administered to a tumor, for example, by direct injection into several different locations within the body of tumor.
30 Alternatively, arteries which serve a tumor may be identified, and the vector injected into such an artery, in order to deliver the vector directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the vector injected directly into the now empty center of the tumor. Within yet another embodiment, the retroviral vector construct may be directly administered to the surface
35 of the tumor, for example, by application of a topical pharmaceutical composition

containing the retroviral vector construct, or preferably, a recombinant retroviral particle.

Within another aspect of the present invention, methods are provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a retroviral vector construct which directs the expression of at least one anti-tumor agent, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited by immune responses generated against the gene-modified tumor cell. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known methods (*see* U.S. Serial No. 08/032,846). Examples of compounds or molecules which act as anti-tumor agents include immune accessory molecules, cytotoxic genes, and antisense sequences as discussed above (*see also* U.S. Serial No. 08/032,846).

Cells may be removed from a variety of locations including, for example, from a selected tumor. In addition, within other embodiments of the invention, a vector construct may be inserted into non-tumorigenic cells, including for example, cells from the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see*, for example, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be contacted with the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal, preferably to or within the vicinity of a tumor. Within one embodiment of the present invention, subsequent to removing tumor cells from a warm-blooded animal, a single cell suspension may be generated by, for example, physical disruption or proteolytic digestion. In addition, division of the cells may be increased by addition of various factors such as melanocyte stimulating factor for melanomas or epidermal growth factor for breast carcinomas, in order to enhance uptake, genomic integration and expression of the recombinant viral vector.

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized to inhibit the growth of selected tumor cells in another, allogeneic, animal. In such a

case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991).

5 The above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation.

10 As noted above, within certain aspects of the present invention, several anti-tumor agents may be administered either concurrently or sequentially, in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, within one embodiment of the invention, an anti-tumor agent such as γ -IFN may be co-administered or sequentially administered to a warm-blooded animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or
15 destroy a pathogenic agent. Such therapeutic compositions may be administered directly utilizing a single vector construct which directs the expression of at least two anti-tumor agents, or, within other embodiments, expressed from independent vector constructs. Alternatively, one anti-tumor agent (*e.g.*, γ -IFN) may be administered utilizing a vector construct, while other tumor agents (*e.g.*, IL-2) are administered directly (*e.g.*, as a
20 pharmaceutical composition intravenously).

 Within a particularly preferred embodiment, retroviral vector constructs which deliver and express both a γ -IFN gene and another gene encoding IL-2, may be administered to the patient. In such constructs, one gene may be expressed from the retrovector LTR and the other may utilize an additional transcriptional promoter found
25 between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of γ -IFN. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

30 Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease (*see, e.g.*, U.S. Serial Nos. 08/104,424; 08/102,132, 07/948,358; 07/965,084), for suppressing graft rejection, (*see* U.S. Serial No. 08/116,827), for suppressing an immune response (*see* U.S. Serial No. 08/116,828), and
35 for suppressing an autoimmune response (*see* U.S. Serial No. 08/116,983).

As will be understood by one of ordinary skill in the art given the disclosure provided herein, any of the retroviral vector constructs described herein may be delivered not only as a recombinant viral particle, but as direct nucleic acid vectors. Such vectors may be delivered utilizing any appropriate physical method of gene transfer, including for example, those which have been discussed above.

5 The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE 1

CONSTRUCTION OF RETROVECTOR BACKBONES

A. Preparation of a Retroviral vector construct That Does Not Contain an Extended Packaging Sequence (Ψ)

5 This example describes the construction of a retroviral vector construct using site-specific mutagenesis. Within this example, a MoMLV retroviral vector construct is prepared wherein the packaging signal " Ψ " of the retrovector is terminated at basepair 617 of SEQ ID NO: 1, thereby eliminating the ATG start of *gag*. Thus, no
10 crossover can occur between the retroviral vector construct and the *gag/pol* expression cassette which is described below in Example 3.

Briefly, pMLV-K (Miller, *J. Virol* 49:214-222, 1984 - an infectious clone derived from pMLV-1 Shinnick et al., *Nature*, 293:543-548, 1981) is digested with *Eco57I*; and a 1.9kb fragment is isolated. (*Eco57I* cuts upstream from the 3' LTR, thereby removing all *env* coding segments from the retroviral vector construct.) The
15 fragment is then blunt ended with T4 polymerase (New England Biolabs), and all four deoxynucleotides, and cloned into the *EcoRV* site of phagemid pBluescript II KS+ (Stratagene, San Diego, Calif.). This procedure yields two constructs, designated pKS2+Eco57I-LTR(+) (Figure 1) and pKS2+Eco57I-LTR(-) (Figure 2), which are
20 screened by restriction analysis. When the (+) single stranded phagemid is generated, the sense sequence of MoMLV is isolated.

A new *EcoRI* site is then created in construct pKS2+Eco57I-LTR(+) in order to remove the ATG start codon of *gag*. In particular, an *EcoRI* site is created using the single stranded mutagenesis method of Kunkle (*PNAS* 82:488, 1985).
25 pKS2+Eco57I-LTR(+) is a pBluescript™ II + phagemid (Stratagene, San Diego, Calif.) containing an *Eco57I* fragment from pMLV-K. It includes the MoMLV LTR and downstream sequence to basepair 1378. When single stranded phagemid is generated the sense sequence of MoMLV is isolated. The oligonucleotide, 5'-GGT AAC AGT CTG GCC CGA ATT CTC AGA CAA ATA CAG (SEQ ID NO: 2), is created and used
30 to generate an *EcoRI* site at basepairs 617-622. This construct is designated pKS2+LTR-EcoRI (Figure 3).

B. Substitution of Nonsense Codons in the Extended Packaging Sequence (Ψ +))

35 This example describes modification of the extended packaging signal (Ψ +) by site-specific mutagenesis. In particular, the modification will substitute a stop

codon, TAA, at the normal ATG start site of *gag* (position 631-633 of SEQ ID NO: 1), and an additional stop codon TAG at position 637-639 of SEQ ID NO: 1.

Briefly, an *Eco57I* - *EcoRI* fragment (MoMLV basepairs 7770 to approx. 1040) from pN2 (Amentano et al., J. Virol. 61:1647-1650, 1987) is first cloned into
5 pBluescript II KS+ phagemid at the *SacII* and *EcoRI* sites (compatible). Single stranded phagemid containing antisense MoMLV sequence, is generated using helper phage M13K07 (Stratagene, San Diego, Calif.). The oligonucleotide 5'-CTG TAT TTG TCT GAG AAT TAA GGC TAG ACT GTT ACC AC (SEQ ID NO: 3) is synthesized, and utilize according to the method of Kunkle as described above, in order to modify the
10 sequence within the Ψ region to encode stop codons at nucleotides 631-633 and 637-639.

C. Removal of Retroviral Packaging Sequence Downstream from the 3' LTR

15 Retroviral packaging sequence which is downstream from the 3' LTR is deleted essentially as described below. Briefly, pKS2+Eco57I-LTR(-) (Figure 2) is digested with *BalI* and *HincII*, and relegated excluding the *BalI* to *HincII* DNA which contains the packaging region of MoMLV.

20 D. Construction of Vector Backbones

Constructs prepared in sections A and C above, or alternatively from sections B and C above, are combined with a plasmid vector as described below, in order to create a retrovector backbone containing all elements required *in cis*, and excluding all sequences of 8 nucleic acids or more contained in the retroviral portion of the *gag-pol*
25 and *env* expression elements (see Examples 3 and 4).

1. Parts A and C are combined as follows: The product of A is digested with *NheI* and *EcoRI*, and a 1034 basepair fragment containing the LTR and minimal Ψ is isolated. The fragment is ligated into the product of part C at the unique (compatible) restriction sites *SpeI* and *EcoRI*. The resultant construct is designated pR1
30 (Figure 4)

2. Parts B and C are combined as follows: The product of B is digested with *NheI* and *EcoRI* and a 1456 basepair fragment containing the LTR and modified Ψ^+ region is isolated. The fragment is ligated into the product of C at the unique (compatible) restriction sites *SpeI* and *EcoRI*. The resultant construct is
35 designated pR2 (Figure 5).

EXAMPLE 2

INSERTION OF A GENE OF INTEREST INTO PR1 AND PR2

5 This example describes the insertion of a gene of interest, gp120, gp41, and rev along with a selectable marker into either pR1 or pR2. Briefly, the sequence encoding gp120, gp41 and rev is taken from construct pKT1 (Figure 6; *see also* Chada et al., *J. Vir.* 67:3409-3417, 1993); note that this vector is also referred to as N2IIIBenv. In particular, pKT1 is first digested at the unique *Asu*II site (position 5959). The ends
10 are blunted, and an *Xho* I linker is ligated at that site. (New England Biolabs). The construct is then digested with *Xho* I, and a 4314 bp fragment containing HIV envelope (gp120 and gp41), rev, SV40 early promoter and G418 resistance genes is isolated.

pR1 or pR2 is digested at the unique *Eco* R1 restriction site, blunted, and *Sal* I linkers (New England Biolabs) are ligated in. The 4314 bp KT1 fragment is then
15 ligated into pR1 or pR2 at the new *Sal* I sites, and the correct orientation is determined (*see* Figures 7 and 8). In both of these constructs, (pR1-HIVenv and pR2-HIVenv) the HIV genes are expressed from the MLV LTR, and G418 resistance is expressed from the SV40 promoter.

EXAMPLE 3

CONSTRUCTION OF GAG-POL EXPRESSION CASSETTES

A. Construction of an Expression Cassette Backbone, pHCMU-PA

A vector is first created in order to form the backbone for both the
25 *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK- phagemid (Stratagene, San Diego, Calif.; GenBank accession number 52324; referred to as "SK-") is digested with *Spe*I and blunt ended with Klenow. A blunt end *Dra*I fragment of SV40 (Fiers et al., "Complete nucleotide sequence of SV40 DNA" *Nature* 273:113-120, 1978) from *Dra*I (bp 2366) to *Dra*I (bp2729) is then inserted into SK-, and a construct isolated in
30 which the SV40 late polyadenylation signal is oriented opposite to the LacZ gene of SK-. This construct is designated SK-SV40A.

A Human Cytomegalovirus Major Immediate Early Promoter ("HCMV-IE"; Boshart et al., *Cell* 41:521-530, 1985) (*Hinc*II, bp 140, to *Eag*I, bp814) is isolated after digestion with *Hinc*II and *Eag*I, and the *Eag*I site blunt ended. The 674 blunt
35 ended fragment is ligated into SK-SV40A. The final construct, designated pHCMV-PA is then isolated (*see* Figure 11). This construct contains the HCMV promoter oriented

in opposite orientation to the LacZ gene, and upstream from the late polyadenylation signal of SV40.

B. Creation of New Codons for the 5' Gag

5 This example describes *gag/pol* expression cassettes that lack non-coding sequences upstream from the *gag* start, thereby reducing recombination potential between the *gag-pol* expression element and Ψ^+ sequence of a retroviral vector construct, and inhibiting co-packaging of the *gag-pol* expression element along with the retrovector. In order to construct such an expression cassette, 448 bp of DNA is
10 synthesized with the following features: 5' ATATATATATATCGAT(*Clai* site)ACCATG(start codon, position 621) (SEQ ID NO: 4), followed by 410 bp encoding 136+ amino acid residues using alternative codons (*see* Figures 9 and 10), followed by GGCGCC(*NarI* site)AAACCTAAAC 3' (SEQ ID NO: 5).

Briefly, each of oligos 15 through 24 (set forth below in Table 1) are
15 added to a PCR reaction tube such that the final concentration for each is 1 μ M. Oligos 25 and 26 are added to the tube such that the final concentration for each is 3 μ M. 1.2 μ L of 2.5 mM stock deoxynucleotide triphosphates (dG, dA, dT, dC) are added to the tube. 5 μ L of 10X PCR buffer (Perkin Elmer). Water is added to a final volume of 50 μ L. Wax beads are added and melted over the aqueous layer at 55°C and then cooled to
20 22°C. A top aqueous layer is added as follows: 5 μ L 10X PCR buffer, 7.5 μ L dimethylsulfoxide, 1.5 μ L Taq polymerase (Perkin-Elmer) and 36 μ L water. Forty cycles of PCR are then performed as follows: 94°C, 30 seconds; 56°C, 30 seconds; and 72°C, 30 seconds. The PCR product is stored at -20°C until assembly of the *gag/pol* expression cassette.

25

Table 1

SEQ. ID. No.	Sequence
15	5' ATA TAT ATA TAT CGA TAC CAT GGG GCA AAC CGT GAC TAC CCC TCT GTC CCT CA C ACT GGC CCA A 3'
16	5' TTG ATT ATG GGC AAT TCT TTC CAC GTC CTT CCA ATG GCC CAG TGT GAG GGA C 3'
17	5' AGA ATT GCC CAT AAT CAA AGC GTG GAC GTC AAA AAA CGC AGG TGG GT G ACA TTT TGT AGC GCC GAG TGG CCC 3'

- 18 5' AAG TTC CAT CCC TAG GCC AGC CAA CAT TGA ATG TGG GCC ACT CGG
CGC
TAC A 3'
- 19 5' GGC CTA GGG ATG GAA CTT TCA ATC GCG ATC TGA TTA CTC AAG TGA AA
A TTA AAG TGT TCA GCC CCG GAC CCC 3'
- 20 5' GTG ACA ATA TAA GGA ACT TGA TCG GGA TGG CCG TGG GGT CCG GGG
CTG
AAC A 3'
- 21 5' AGT TCC TTA TAT TGT CAC ATC GGA GGC TCT CGC TTT CGA TCC ACC
ACC TTG GGT GAA ACC ATT CGT GCA TCC 3'
- 22 5' AGG AGC GCT GGG TGG GAG GGG TGG AGG TGG TTT GGG ATG CAC GAA
TGG TTT C 3'
- 23 5' CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC
ACA CCA CCC AGG AGC AGC TTG TAC CCT 3'
- 24 5' GTT TAG GTT TGG CGC CGA GGC TGG GGG TCA GAG CAG GGT ACA AGC
TGC
TCC T 3'
- 25 5' ATA TAT ATA TAT CGA TAC C 3'
- 26 5' GTT TAG GTT TGG CGC CGA GG 3'

C. Creation of a New 3' End for *Pol*

In order to prepare a *gag/pol* expression cassette which expresses full length *gag/pol*, pCMV*gag/pol* is constructed. Briefly, MoMLV sequence from *Pst*I (BP567) to *Nhe*I (bp 7847) is cloned into the *Pst*I-*Xba*I sites of pUC19 (New England Biolabs). The resultant intermediate is digested with *Hind*III and *Xho*I, and a 1008 bp fragment containing the *gag* leader sequence is isolated. The same intermediate is also digested with *Xho*I and *Sca*I, and a 4312 bp fragment containing the remaining *gag* and *pol* sequences is isolated. The two isolated fragments are then cloned into the *Hind*III and *Sma*I sites of pHCMV-PA, described above. The resultant construct, designated CMV *gag/pol* (Figure 12) expresses MoMLV *gag* and *pol* genes.

In order to truncate the 3' end of the *pol* gene found in pCMV *gag-pol*, a 5531 basepair *Sna*BI - *Xma*I fragment containing a portion of the CMV IE promoter and all of *gag-pol* except the final 28 codons, is isolated from pCMV *gag-pol*. This fragment is cloned into the *Sna*BI and *Xma*I sites of pHCMV-PA. This construct expresses five new amino acids at the carboxy-terminus (Ser-Lys-Asn-Tyr-Pro) (SEQ ID NO: 6) (pCMV gpSma).

Alternatively, these five amino acids may be eliminated by digesting pCMVgp *Sma*I with *Sma*I and adding an *Nhe*I (termination codons in three phases) linker (5' - CTA GCT AGC TAG SEQ ID NO: 14; New England Biolabs) at the end of the truncated *pol* sequence. This construct is designated pCMV gp *Nhe*. Both of these
 5 constructs eliminates potential crossover between *gag/pol* and *env* expression cassettes.

D. Gag-Pol Expression Cassette

Parts B and C from above are combined to provide an expression vector containing a CMV IE promoter, *gag-pol* sequence starting from the new *Cla*I site
 10 (followed by ACC ATG and 412 bp of alternative or "wobble" *gag* coding sequence) and terminating at the *Sma*I site (MoMLV position 5750) followed by an SV40 polyadenylation signal, essentially as described below. Briefly, the approximately 451 bp double stranded wobble fragment from part A is ligated into pCRTMII TA cloning vector (Invitrogen Corp.). The wobble PCR product naturally contains a 3' A-overhang at each
 15 end, allowing for cloning into the 3' T-overhang of pCRTMII. The 422 bp *Cla*I -*Nar*I wobble fragment from the pCRTMII clone is removed and is ligated into the *Cla*I (Position 679, Figure pCMV gp *Sma*) and *Nar*I (Position 1585) sites of pCMVgp *Sma*I (Part B) (or pCMV gp *Nhe*). (The *Cla*I site at position 5114 is methylated and not cut with *Cla*I). The product of that ligation is digested with *Nar*I, and the MLV-K *Nar*I
 20 fragment (positions 1035 to 1378) is inserted (SEQ ID NO: 1). This construct is designated pCMVgp -X (Figure 14).

EXAMPLE 4

CONSTRUCTION OF *ENV* EXPRESSION CASSETTES

25

A. Creation of a New 5' *Eag*I Restriction Site

Starting with an *Eag*I- *Eco*RI 626 bp subfragment from a 4070A amphotropic envelope (Chattopodhyay et al., *J. Vir.* 39:777, 1981; GenBank accession # MLV4070A, and #MLVENVC; SEQ ID NO: 12) cloned in a pBluescript II Ks+ vector
 30 (containing the start codon), site directed mutagenesis is performed upstream of the translation start site in order to change ACCATCCTCTGGACGGACATG... (SEQ ID NO: 7; positions 20 - 40 of Genebank sequence # MLVENVC) to ACCCGGCCGTGGACGGACATG... (SEQ ID NO: 8) and create a new *Eag*I site at position 23. This modification allows cloning of the amphotropic envelope sequence
 35 into an expression vector eliminating upstream 4070A sequence homologous to the *gag-pol* expression element as described in Example 2A.

B. Creation of a New 3' End for Env

A new 3' end of the envelope expression element is created by terminating the sequence which encodes the R-peptide downstream from the end of the transmembrane region (p15E). Briefly, construct pHCMV-PA, described above, is first modified by digestion with *NcoI* (position 1097), blunted and relegated to obliterate the overlapping Bluescript *EagI* site at the same position. pCMV Envam-Eag-X-less is then constructed by digesting the modified pHCMV-PA with *EagI* (position 671 and *SmaI* (position 712) and ligating in two fragments. The first is an *EagI-NcoI* fragment from 4070A (positions 1-1455) (SEQ ID NO: 12). The second is an MLV-K envelope fragment, *NcoI-PvuII* (positions 7227-7747) (SEQ ID NO: 12). The resultant construct from the three-way ligation contains the HCMV promoter followed by the SU (GP70) coding sequence of the 4070A envelope, the TM (p15e) coding sequence of MoMLV, and sequence encoding 8 residues of the R-peptide. In addition, this envelope expression cassette (pCMV Env am-Eag-X-less) (Figure 18) shares no sequence with crossless retrovector backbones described in Example 1.

C. Envelope Expression Element

Parts A and B from above are combined to complete an amphotropic expression element containing the CMV promoter, 4070A SU, MoMLV TM and SV40 polyadenylation signal in a Bluescript SK- plasmid vector. This construct is called pCMVenv-X (Figure 15). Briefly, the construct described in part A with a new *EagI* restriction site is digested with *EagI* and *XhoI*, and a 571 bp fragment is isolated. pCMV Envam-Eag-X-less (from part B) is digested with *KpnI* and *EagI* and the 695 bp fragment is reserved. pCMV Envam-Eag-X-less (from part B) is digested with *KpnI* and *XhoI* and the 4649 bp fragment is reserved. These two fragments are ligated together along with the 571 bp *EagI* to *XhoI* fragment digested from the PCR construct from part A. pCMVenv-X shares no sequence with crossless retrovector backbones nor the *gag-pol* expression element pCMVgp-X.

EXAMPLE 5

FUNCTIONALITY TESTS FOR *GAG-POL* AND *ENV* EXPRESSION CASSETTES

Rapid tests have been developed in order to ensure that the *gag-pol* and *env* expression cassettes are biologically active. The materials for these tests consist of a cell line used for transient expression (typically 293 cells, ATCC #CRL 1573), a target

cell line sensitive to infection (typically HT 1080 cells, ATCC #CCL 121) and either pRgpNeo (Figure 16) or pLARNL (Emi et al., *J. Virol* 65:1202-1207, 1991). The two later plasmids express rescuable retrovectors that confer G418 resistance and also express *gag-pol*, in the case of RgpNeo or *env*, in the case of pLARNL. For convenience, the organization of RgpNeo (Figure 16) is set forth below.

In order to test expression cassettes such as pCMVgp-X for functionality of *gag/pol*, the plasmid is co-transfected with pLARNL at a 1:1 ratio into 293 cells. After 12 hours, the media is replaced with normal growth media. After an additional 24 hours, supernatant fluid is removed from the 293 cells, filtered through a 0.45 μ m filter, and placed on HT 1080 target cells. Twenty-four hours after that treatment, the media is replaced with growth media containing 800 ug/ml G418. G418 resistant colonies are scored after one week. The positive appearance of colonies indicates that all elements are functional and active in the original co-transfection.

For convenience, the organization of RgpNeo (Figure 16) is set forth below:
Position 1 = left end of 5' LTR; Positions 1-6320 = MoMLV sequence from 5'LTR to Sca 1 restriction site; Positions 6321 - 6675 = SV40 early promoter; Positions 6676-8001 = Neo resistance gene from Tn 5 (including prokaryotic promoter); and Positions 8002 - 8606 = pBR origin of replication.

EXAMPLE 6

PACKAGING CELL LINE AND PRODUCER CELL LINE DEVELOPMENT

This example describes the production of packing and producer cell lines utilizing the above described retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes, which preclude the formation of replication competent virus.

Briefly, for amphotropic MoMLV-based retroviral vector constructs, a parent cell line is selected which lacks sequences which are homologous to Murine Leukemia Viruses, such as the dog cell line D-17 (ATCC No. CCL 183). The *gag/pol* expression cassettes are then introduced into the cell by electroporation, along with a selectable marker plasmid such as DHFR (Simonsen et al., *PNAS* 80:2495-2499, 1983). Resistant colonies are then selected, expanded in 6 well plates to confluency, and assayed for expression of *gag/pol* by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with pLARNL.

The highest titer clones are then electroporated with an *env* expression cassette and a selectable marker plasmid such as hygromycin (*see* Gritz and Davies, *Gene* 25:179-188, 1983). Resistant colonies are selected, expanded in 6 well plates to confluency, and assayed for expression of *env* by Western Blots. Clones are also
5 screened for the production of high titer vector particles after transduction with a retroviral vector construct.

Resultant packaging cell lines may be stored in liquid Nitrogen at 10×10^6 cells per vial, in DMEM containing 10% irradiated Fetal Bovine Serum, and 8% DMSO. Further testing may be accomplished in order to confirm sterility, and lack of
10 helper virus production. Preferably, both an S+L- assay and a *Mus dunni* marker rescue assay should be performed in order to confirm a lack of helper virus production.

In order to construct a producer cell line, retroviral vector construct as described above in Example 1 is electroporated into a xenotropic packaging cell line made utilizing the methods described above. After 24-48 hours, supernatant fluid is
15 removed from the xenotropic packaging cell line, and utilized to transduce a second packaging cell line, thereby creating the final producer cell line.

EXAMPLE 7

HELPER DETECTION ASSAY COCULTIVATION, AND MARKER RESCUE

20

This example describes a sensitive assay for the detection of replication competent retrovirus ("RCR"). Briefly, 5×10^5 vector-producing cells are cocultivated with an equal number of *Mus dunni* cells (Lander and Chattopadhyay, *J. Virol.* 52:695, 1984). *Mus dunni* cells are particularly preferred for helper virus detection because they
25 are sensitive to nearly all murine leukemia-related viruses, and contain no known endogenous viruses. At three, six, and nine days after the initial culture, the cells are split approximately 1 to 10, and 5×10^5 fresh *Mus dunni* cells are added. Fifteen days after the initial cocultivation of *Mus dunni* cells with the vector-producing cells, supernatant fluid is removed from cultures, filtered through a 0.45 μ m filter, and
30 subjected to a marker rescue assay.

Briefly, culture fluid is removed from a MdH tester cell line (*Mus dunni* cells containing pLHL (a hygromycin resistance marker retroviral vector; *see* Palmer et al., *PNAS* 84(4):1055-1059, 1987) and replaced with the culture fluid to be tested. Polybrene is added to a final concentration of 4 μ g/ml. On day 2, medium is removed
35 and replaced with 2 ml of fresh DMEM containing 10% Fetal Calf Serum. On day 3, supernatant fluid is removed, filtered, and transferred to HT1080 cells. Polybrene is

added to a final concentration of 4µg/ml. On day 4, medium in the HT1080 cells is replaced with fresh DMEM containing 10% Fetal Calf Serum, and 100 µg/ml hygromycin. Selection is continued on days 5 through 20 until hygromycin resistant colonies can be scored, and all negative controls (*e.g.*, mock infected MdH cells) are dead.

EXAMPLE 8

ASSAY FOR ENCAPSIDATION OF WOBBLE RNA SEQUENCE

This example describes a sensitive assay for the detection of encapsidation of RNA from constructs containing wobble or normal gag sequence. Briefly, a fragment of DNA from a "wobble" *gag/pol* expression cassette (Example 3), containing the CMV promoter and gag sequence to the *Xho*I site (MoMLV position 1561) is ligated to a SV40 neo-3' LTR DNA fragment from N2 (Armentano et al., *supra*) or KT-3 (*see* WO 91/02805 or WO 92/05266). This construct is diagrammatically illustrated in Figure 19A, and is not expected to be encapsidated in packaging cell lines such as DA or HX (*see* WO 92/05266) because it lacks a 5' LTR and primer binding site.

A second construct is also made, similar to the first except that the wobble sequence is replaced by normal *gag* sequence. Similar to the first construct, the RNA transcribed from this DNA is not expected to be encapsidated. This construct is diagrammatically illustrated in Figure 19B.

The above constructs are separately transfected into a packaging cell line. The culture is then assayed for the ability to generate transducible G418-resistant retrovector. Neither construct results in transducible vector.

Cell cultures containing the above constructs are then transduced with the retrovector LHL (*see* Example 7). The cell cultures, after selection, will now generate retrovector conferring hygromycin resistance to target cells. Further, if co-encapsidation is allowed by interaction between LHL RNA and the transcripts from the above constructs, statistically significant RT-mediated recombination can occur resulting in the transfer of G418 resistance to target cells.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration,

various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Respass, James
- (ii) TITLE OF INVENTION: Crossless Retroviral Vectors
- (iii) NUMBER OF SEQUENCES: 26
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Seed & Berry
 - (B) STREET: 6300 Columbia Center; 701 Fifth Avenue
 - (C) CITY: Seattle
 - (D) STATE: Washington
 - (E) COUNTRY: USA
 - (F) ZIP: 98104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: McMasters, David D.
 - (B) REGISTRATION NUMBER: 33,963
 - (C) REFERENCE/DOCKET NUMBER: 930049.424C1
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (206)622-4900
 - (B) TELEFAX: (206)682-6031

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8332 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AACCTCTTG	60
CAGTTGCATC CGACTTGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCTG AGTGATTGAC	120

TACCCGTCAG CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCGGGAGA CCCCTGCCCCA	180
GGGACCACCG ACCCACCACC GGGAGGTAAG CTGGCCAGCA ACTTATCTGT GTCTGTCCGA	240
TTGTCTAGTG TCTATGACTG ATTTTATGCG CCTGCGTCGG TACTAGTTAG CTAAGTAGCT	300
CTGTATCTGG CGGACCCGTG GTGGAAGTGA CGAGTTCGGA ACACCCGGCC GCAACCCTGG	360
GAGACGTCCC AGGGACTTCG GGGGCCGTTT TTGTGGCCCG ACCTGAGTCC AAAAATCCCG	420
ATCGTTTTGG ACTCTTTGGT GCACCCCCCT TAGAGGAGGG ATATGTGGTT CTGGTAGGAG	480
ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGGACCGAA	540
GCCGCGCCGC GCGTCTTGTC TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT	600
TTCTGTATTT GTCTGAGAAT ATGGGCCAGA CTGTTACCAC TCCCTTAAGT TTGACCTTAG	660
GTCAGTGAA AGATGTCGAG CGGATCGCTC ACAACCAGTC GGTAGATGTC AAGAAGAGAC	720
GTTGGGTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG CCGCGAGACG	780
GCACCTTTAA CCGAGACCTC ATCACCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCCGC	840
ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC	900
CTCCCTGGGT CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCTT CCATCCGCCC	960
CGTCTCTCCC CTTGAACCT CCTCGTTCGA CCCCGCCTCG ATCCTCCCTT TATCCAGCCC	1020
TCACTCCTTC TCTAGGCGCC AAACCTAAAC CTCAAGTTCT TTCTGACAGT GGGGGGCCGC	1080
TCATCGACCT ACTTACAGAA GACCCCCCGC CTTATAGGGA CCCAAGACCA CCCCCTTCCG	1140
ACAGGGACGG AAATGGTGGA GAAGCGACCC CTGCGGGAGA GGCACCGGAC CCCTCCCCAA	1200
TGGCATCTCG CCTACGTGGG AGACGGGAGC CCCCTGTGGC CGACTCCACT ACCTCGCAGG	1260
CATTCCCCCT CCGCGCAGGA GGAAACGGAC AGCTTCAATA CTGGCCGTTT TCCTCTTCTG	1320
ACCTTTACAA CTGGAAAAAT AATAACCCTT CTTTTTCTGA AGATCCAGGT AACTGACAG	1380
CTCTGATCGA GTCTGTTCTC ATCACCATC AGCCACCTG GGACGACTGT CAGCAGCTGT	1440
TGGGGACTCT GCTGACCGGA GAAGAAAAAC AACGGGTGCT CTTAGAGGCT AGAAAGGCGG	1500
TGCGGGGCGA TGATGGGCGC CCCACTCAAC TGCCCAATGA AGTCGATGCC GCTTTTCCCC	1560
TCGAGCGCCC AGACTGGGAT TACACCACCC AGGCAGGTAG GAACCACCTA GTCCACTATC	1620
GCCAGTTGCT CCTAGCGGGT CTCCAAAACG CGGGCAGAAG CCCCACCAAT TTGGCCAAGG	1680
TAAAAGGAAT AACACAAGGG CCCAATGAGT CTCCTCGGC CTTCTAGAG AGACTTAAGG	1740

AAGCCTATCG CAGGTACACT CCTTATGACC CTGAGGACCC AGGGCAAGAA ACTAATGTGT	1800
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GCCGTAGGAC AGAGGATGAG CAGAAAGAGA AAGAAAGAGA TCGTAGGAGA CATAGAGAGA	2040
TGAGCAAGCT ATTGGCCACT GTCGTTAGTG GACAGAAACA GGATAGACAG GGAGGAGAAC	2100
GAAGGAGGTC CCAACTCGAT CGCGACCAGT GTGCCTACTG CAAAGAAAAG GGGCACTGGG	2160
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TGACCCTAGA TGACTIONGGA GGTGAGGTC AGGAGCCCCC CCCTGAACCC AGGATAACCC	2280
TCAAAGTCGG GGGGCAACCC GTCACCTTCC TGGTAGATAC TGGGGCCCAA CACTCCGTGC	2340
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GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGTCCCCAGA TGCGGTCCAG	8100
CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCAGGG TGCCCCAAGG ACCTGAAATG	8160
ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC	8220
TGCTCCCCGA GCTCAATAAA AGAGCCCACA ACCCTCACT CGGGGCGCCA GTCCTCCGAT	8280
TGACTGAGTC GCCCGGTAC CCGTGTATCC AATAAACCTT CTTGCAGTTG CA	8332

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTAACAGTC TGGCCCGAAT TCTCAGACAA ATACAG

36

2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGTATTTGT CTGAGAATTA AGGCTAGACT GTTACCAC

38

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATATATAT ATCGATACCA TG

22

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCGCCAAAC CTAAAC

16

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Lys Asn Tyr Pro

5

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCATCCTCT GGACGGACAT G

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCGGCCGT GGACGGACAT G

21

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 449 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 20..439

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATATATATAT ATCGATACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC CTC	52
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu	
1 5 10	
ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC	100
Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser	
15 20 25	
GTG GAC TGC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG	148
Val Asp Cys Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp	
30 35 40	
CCC ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT	196
Pro Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp	
45 50 55	
CTG ATT ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC	244
Leu Ile Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly	
60 65 70 75	
CAT CCC GAT CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC	292
His Pro Asp Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe	
80 85 90	
GAT CCA CCA CCT TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA	340
Asp Pro Pro Pro Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro	
95 100 105	
CCC CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC	388
Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser	
110 115 120	
ACA CCA CCC AGG AGC AGC TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC	436
Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly	
125 130 135	
GCC AACCTAAAC	449
Ala	
140	

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 140 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1           5           10           15
Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Cys Lys Lys
          20           25           30
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
      35           40           45
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
 50           55           60
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
 65           70           75           80
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
          85           90           95
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
      100           105           110
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
      115           120           125
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
      130           135           140

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 420 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..420

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG	GGC	CAG	ACT	GTT	ACC	ACT	CCC	TTA	AGT	TTG	ACC	TTA	GGT	CAC	TGG	48
Met	Gly	Gln	Thr	Val	Thr	Thr	Pro	Leu	Ser	Leu	Thr	Leu	Gly	His	Trp	
1				5					10					15		
AAA	GAT	GTC	GAG	CGG	ATC	GCT	CAC	AAC	CAG	TCG	GTA	GAT	GTC	AAG	AAG	96
Lys	Asp	Val	Glu	Arg	Ile	Ala	His	Asn	Gln	Ser	Val	Asp	Val	Lys	Lys	
			20					25					30			
AGA	CGT	TGG	GTT	ACC	TTC	TGC	TCT	GCA	GAA	TGG	CCA	ACC	TTT	AAC	GTC	144
Arg	Arg	Trp	Val	Thr	Phe	Cys	Ser	Ala	Glu	Trp	Pro	Thr	Phe	Asn	Val	
			35				40					45				
GGA	TGG	CCG	CGA	GAC	GGC	ACC	TTT	AAC	CGA	GAC	CTC	ATC	ACC	CAG	GTT	192
Gly	Trp	Pro	Arg	Asp	Gly	Thr	Phe	Asn	Arg	Asp	Leu	Ile	Thr	Gln	Val	
	50					55					60					
AAG	ATC	AAG	GTC	TTT	TCA	CCT	GGC	CCG	CAT	GGA	CAC	CCA	GAC	CAG	GTC	240
Lys	Ile	Lys	Val	Phe	Ser	Pro	Gly	Pro	His	Gly	His	Pro	Asp	Gln	Val	
	65				70				75					80		
CCC	TAC	ATC	GTG	ACC	TGG	GAA	GCC	TTG	GCT	TTT	GAC	CCC	CCT	CCC	TGG	288
Pro	Tyr	Ile	Val	Thr	Trp	Glu	Ala	Leu	Ala	Phe	Asp	Pro	Pro	Pro	Trp	
				85				90						95		
GTC	AAG	CCC	TTT	GTA	CAC	CCT	AAG	CCT	CCG	CCT	CCT	CTT	CCT	CCA	TCC	336
Val	Lys	Pro	Phe	Val	His	Pro	Lys	Pro	Pro	Pro	Pro	Leu	Pro	Pro	Ser	
			100					105					110			
GCC	CCG	TCT	CTC	CCC	CTT	GAA	CCT	CCT	CGT	TCG	ACC	CCG	CCT	CGA	TCC	384
Ala	Pro	Ser	Leu	Pro	Leu	Glu	Pro	Pro	Arg	Ser	Thr	Pro	Pro	Arg	Ser	
		115					120					125				
TCC	CTT	TAT	CCA	GCC	CTC	ACT	CCT	TCT	CTA	GGC	GCC					420
Ser	Leu	Tyr	Pro	Ala	Leu	Thr	Pro	Ser	Leu	Gly	Ala					
	130					135					140					

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
1 5 10 15

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
20 25 30

Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val

35	40	45
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val		
50	55	60
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val		
65	70	75
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp		
85	90	95
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser		
100	105	110
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser		
115	120	125
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala		
130	135	140

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2001 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCCGACACC CAGAGTGGAC CATCCTCTGG ACGGACATGG CGCGTTCAAC GCTCTCAAAA	60
CCCCCTCAAG ATAAGATTAA CCCGTGGAAG CCCTTAATAG TCATGGGAGT CCTGTTAGGA	120
GTAGGGATGG CAGAGAGCCC CCATCAGGTC TTTAATGTAA CCTGGAGAGT CACCAACCTG	180
ATGACTGGGC GTACCGCCAA TGCCACCTCC CTCCTGGGAA CTGTACAAGA TGCCTTCCCA	240
AAATTATATT TTGATCTATG TGATCTGGTC GGAGAGGAGT GGGACCCTTC AGACCAGGAA	300
CCGTATGTCG GGTATGGCTG CAAGTACCCC GCAGGGAGAC AGCGGACCCG GACTTTTGAC	360
TTTTACGTGT GCCCTGGGCA TACCGTAAAG TCGGGGTGTG GGGGACCAGG AGAGGGCTAC	420
TGTGGTAAAT GGGGGTGTGA AACCACCGGA CAGGCTTACT GGAAGCCAC ATCATCGTGG	480
GACCTAATCT CCCTTAAGCG CGGTAACACC CCCTGGGACA CGGGATGCTC TAAAGTTGCC	540
TGTGGCCCCT GCTACGACCT CTCAAAGTA TCCAATTCCT TCCAAGGGGC TACTCGAGGG	600
GGCAGATGCA ACCCTCTAGT CCTAGAATTC ACTGATGCAG GAAAAAAGGC TAACTGGGAC	660

GGGCCCAAAT CGTGGGGACT GAGACTGTAC CGGACAGGAA CAGATCCTAT TACCATGTTC	720
TCCCTGACCC GGCAGGTCCT TAATGTGGGA CCCCAGAGTCC CCATAGGGCC CAACCCAGTA	780
TTACCCGACC AAAGACTCCC TTCCTACCA ATAGAGATTG TACCGGCTCC ACAGCCACCT	840
AGCCCCCTCA ATACCAGTTA CCCCCCTTCC ACTACCAGTA CACCCTCAAC CTCCCCTACA	900
AGTCCAAGTG TCCCACAGCC ACCCCCAGGA ACTGGAGATA GACTACTAGC TCTAGTCAAA	960
GGAGCCTATC AGGCGCTTAA CCTCACCAAT CCCGACAAGA CCCAAGAATG TTGGCTGTGC	1020
TTAGTGTCGG GACCTCCTTA TTACGAAGGA GTAGCGGTCTG TGGGCACTTA TACCAATCAT	1080
TCCACCGCTC CGGCCAACTG TACGGCCACT TCCCAACATA AGCTTACCCT ATCTGAAGTG	1140
ACAGGACAGG GCCTATGCAT GGGGGCAGTA CCTAAAACTC ACCAGGCCTT ATGTAACACC	1200
ACCCAAAGCG CCGGCTCAGG ATCCTACTAC CTTGCAGCAC CCGCCGGAAC AATGTGGGCT	1260
TGCAGCACTG GATTGACTCC CTGCTTGTCC ACCACGGTGC TCAATCTAAC CACAGATTAT	1320
TGTGTATTAG TTGAACTCTG GCCCAGAGTA ATTTACCACT CCCCCGATTA TATGTATGGT	1380
CAGCTTGAAC AGCGTACCAA ATATAAAAGA GAGCCAGTAT CATTGACCCT GGCCCTTCTA	1440
CTAGGAGGAT TAACCATGGG AGGGATTGCA GCTGGAATAG GGACGGGGAC CACTGCCTTA	1500
ATTAAACCC AGCAGTTTGA GCAGCTTCAT GCCGCTATCC AGACAGACCT CAACGAAGTC	1560
GAAAAGTCAA TTACCAACCT AGAAAAGTCA CTGACCTCGT TGTCTGAAGT AGTCCTACAG	1620
AACCGCAGAG GCCTAGATTT GCTATTCTTA AAGGAGGGAG GTCTCTGCGC AGCCCTAAAA	1680
GAAGAATGTT GTTTTTATGC AGACCACACG GGGCTAGTGA GAGACAGCAT GGCCAAATTA	1740
AGAGAAAGGC TTAATCAGAG ACAAAAATA TTTGAGACAG GCCAAGGATG GTTCGAAGGG	1800
CTGTTTAATA GATCCCCCTG GTTTACCACC TTAATCTCCA CCATCATGGG ACCTCTAATA	1860
GTA CTCTTAC TGATCTTACT CTTTGGACCT TGCATTCTCA ATCGATTGGT CCAATTTGTT	1920
AAAGACAGGA TCTCAGTGGT CCAGGCTCTG GTTTTGA CTC AGCAATATCA CCAGCTAAAA	1980
CCCATAGAGT ACGAGCCATG A	2001

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAGCTAGCT AG

12

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATATATAT ATCGATACCA TGGGGCAAAC CGTGACTACC CCTCTGTCCC TCACACTGGC

60

CCAA

64

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTGATTATGG GCATTCTTT CCACGTCCTT CCAATGGCCC AGTGTGAGGG A

51

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGAATTGCCC ATAATCAAAG CGTGGACGTC AAAAAACGCA GGTGGGTGAC ATTTTGTAGC

60

GCCGAGTGGC CC

72

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 52 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTCCATC CCTAGGCCAG CCAACATTGA ATGTGGGCCA CTCGGCGCTA CA 52

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 71 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCCTAGGGA GGAAC TTCA ATCGCGATCT GATTACTCAA GTGAAAATTA AAGTGTT CAG 60
CCCCGGACCC C 71

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 52 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTGACAATAT AAGGA ACTTG ATCGGGATGG CCGTGGGGTC CGGGGCTGAA CA 52

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 72 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTCCTTAT ATTGTCACAT CGGAGGCTCT CGCTTTCGAT CCACCACCTT GGGTGAAACC 60
ATTCGTGCAT CC 72

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

AGGAGCGCTG GGTGGGAGGG GTGGAGGTGG TTTGGGATGC ACGAATGGTT TC 52

(2) INFORMATION FOR SEQ ID NO:23

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCCACCCA GCGCTCCTAG CCTGCCCTTG GAGCCCCAC GAAGCACACC ACCCAGGAGC 60
AGCTTGTAAC CT 72

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTTAGGTTT GGCGCCGAGG CTGGGGGTCA GAGCAGGGTA CAAGCTGCTC CT 52

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATATATATAT ATCGATACC

19

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTTAGGTTT GGCGCCGAGG

20

Claims

1. A retroviral vector construct comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein said vector construct lacks *gag/pol* and *env* coding sequences.
2. The retroviral vector construct according to claim 1 wherein said vector construct lacks an extended packaging signal.
3. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral nucleic acid sequence upstream of said 5' LTR.
4. The retroviral vector construct according to claim 3 wherein said construct lacks an *env* coding sequence upstream of said 5' LTR.
5. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral packaging signal sequence downstream of said 3' LTR.
6. The retroviral vector construct according to any one of claims 1 to 5 wherein said retrovector is constructed from a retrovirus selected from the group consisting of amphotropic, ecotropic, xenotropic or polytropic viruses.
7. The retroviral vector construct according to any one of the claims 1 to 5 wherein said retrovector is constructed from a Murine Leukemia Virus.
8. The retroviral vector construct according to any one of claims 1 to 5, wherein said heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 2, 3, 4, 5, 6, 7 and 8.
9. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a gene encoding a cytotoxic protein.
10. The retroviral vector construct according to claim 9 wherein said cytotoxic protein is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera

toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

11. The retroviral vector construct according to claim 8 wherein said heterologous sequence is an antisense sequence.

12. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immune accessory molecule.

13. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, and IL-13.

14. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon.

15. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of ICAM-1, ICAM-2, β -microglobulin, LFA3, HLA class I and HLA class II molecules.

16. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes a gene product that activates a compound with little or no cytotoxicity into a toxic product.

17. The retroviral vector construct according to claim 16 wherein said gene product is selected from the group consisting of HSVTK and VZVTK.

18. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a ribozyme.

19. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a replacement gene.

20. The retroviral vector construct according to claim 19 wherein said replacement gene encodes a protein selected from the group consisting of Factor VIII, ADA, HPRT, CF and the LDL Receptor.

21. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

22. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

23. The *gag/pol* expression cassette according to claim 22 wherein the 5' terminal end of said *gag/pol* gene lacks a retroviral packaging signal sequence.

24. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *gag/pol* gene has been deleted without affecting the biological activity of integrase.

25. The *gag/pol* expression cassette according to claim 24 wherein a 5' terminal end of said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

26. The *gag/pol* expression cassette according to claim 24 wherein said *gag/pol* gene lacks a retroviral packaging signal sequence.

27. The *gag/pol* expression cassette according to claims 24 to 26 wherein said 3' terminal end has been deleted upstream of nucleotide 5751 of Sequence ID No. 1.

28. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said promoter is a heterologous promoter.

29. The *gag/pol* expression cassette according to claim 28 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

30. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

31. The *gag/pol* expression cassette according to claim 30 wherein said heterologous polyadenylation sequence is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

32. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said expression cassette does not co-encapsidate with a replication competent virus.

33. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 consecutive retroviral nucleotides are included upstream of said *env* gene.

34. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein said *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene.

35. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *env* gene has been deleted without effecting the biological activity of *env*.

36. The *env* expression cassette according to claim 35 wherein said 3' terminal end of said gene has been deleted such that a complete R peptide is not produced by said expression cassette.

37. The *env* expression cassette according to claim 36 wherein said *env* gene is derived from a type C retrovirus, and wherein the 3' terminal end has been deleted such that said *env* gene includes less than 18 nucleic acids which encode said R peptide.

38. The *env* expression cassette according to claim 36 wherein said 3' terminal end has been deleted downstream from nucleotide 7748 of Sequence ID. No. 1.

39. The *env* expression cassette according to any one of claims 33 to 38 wherein said promoter is a heterologous promoter.

40. The *env* expression cassette according to claim 39 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

41. The *env* expression cassette according to any one of claims 33 to 38 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

42. The *env* expression cassette according to claim 41 wherein said heterologous polyadenylation is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

43. A packaging cell line, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein said *gag/pol* expression cassette lacks a consecutive sequence of greater than 8 consecutive nucleotides which are found in said *env* expression cassette.

44. A packaging cell line, comprising a *gag/pol* expression cassette according to claims 22 to 32, and an *env* expression cassette.

46. A packaging cell line, comprising a *gag/pol* expression cassette, and an *env* expression cassette according to claims 33 to 42.

46. A producer cell line, comprising a packaging cell line according to any one of claims 43 to 45, and a retroviral vector construct.

47. The producer cell line according to claim 46 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

48. A producer cell line, comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein said *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 8 nucleotides in common.

49. A method of producing a packaging cell, comprising:
(a) introducing a *gag/pol* expression cassette according to claims 22 to 32 into an animal cell;

- (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*;
- (c) introducing an *env* expression cassette into said selected cell; and
- (d) selecting a cell which expresses high levels of *env*, and thereby producing said packaging cell.

50. A method of producing a packaging cell, comprising:

- (a) introducing an *env* expression cassette according to claims 33 to 42 into an animal cell;
- (b) selecting a cell which expresses high levels of *env*;
- (c) introducing a *gag/pol* expression cassette into said selected cell; and
- (d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby producing said packaging cell.

51. A method of producing recombinant retroviral particles, comprising introducing a retroviral vector construct into packaging cell line according to claim 49 or 50.

52. The method according claim 51 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

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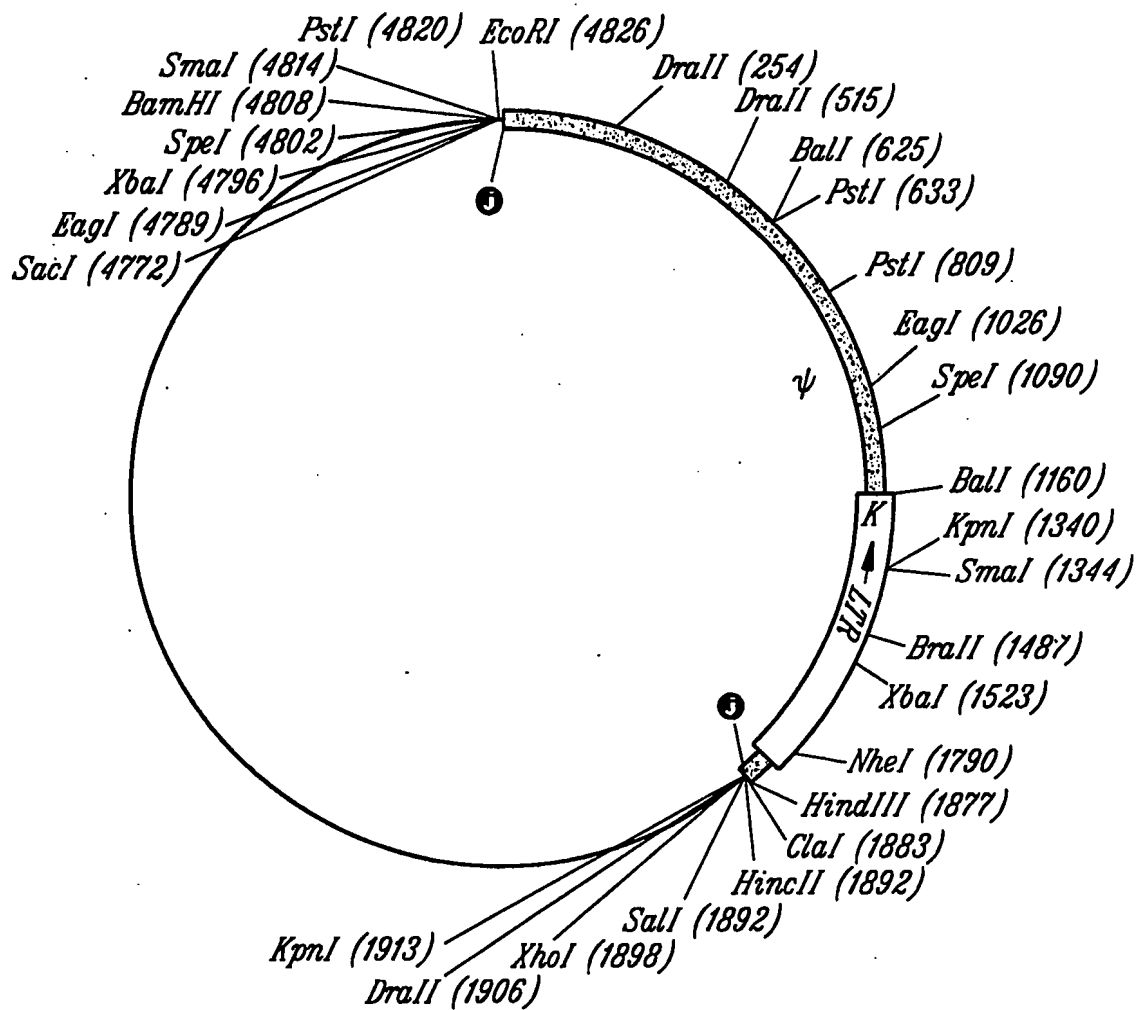


Fig. 1

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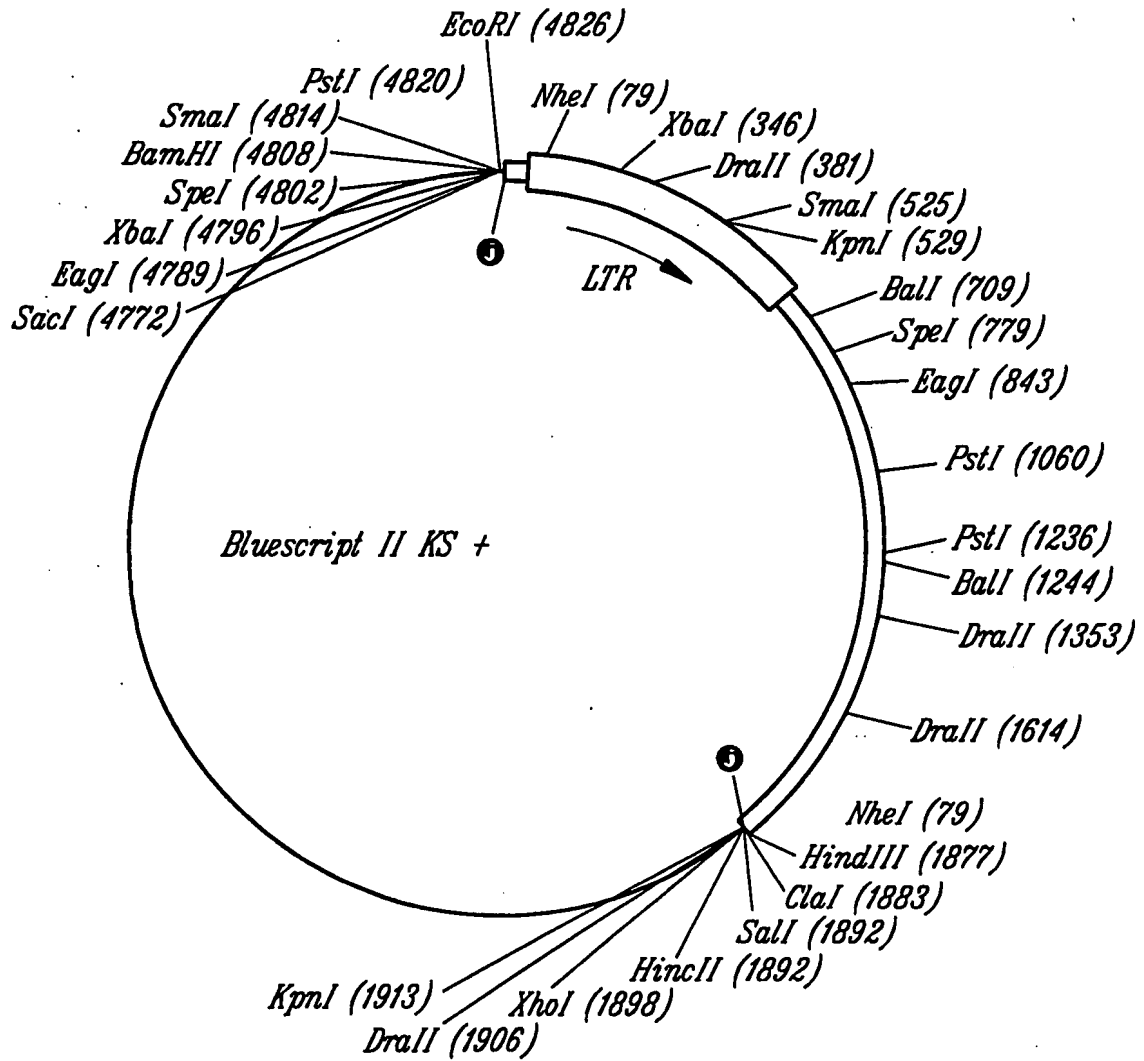


Fig. 2

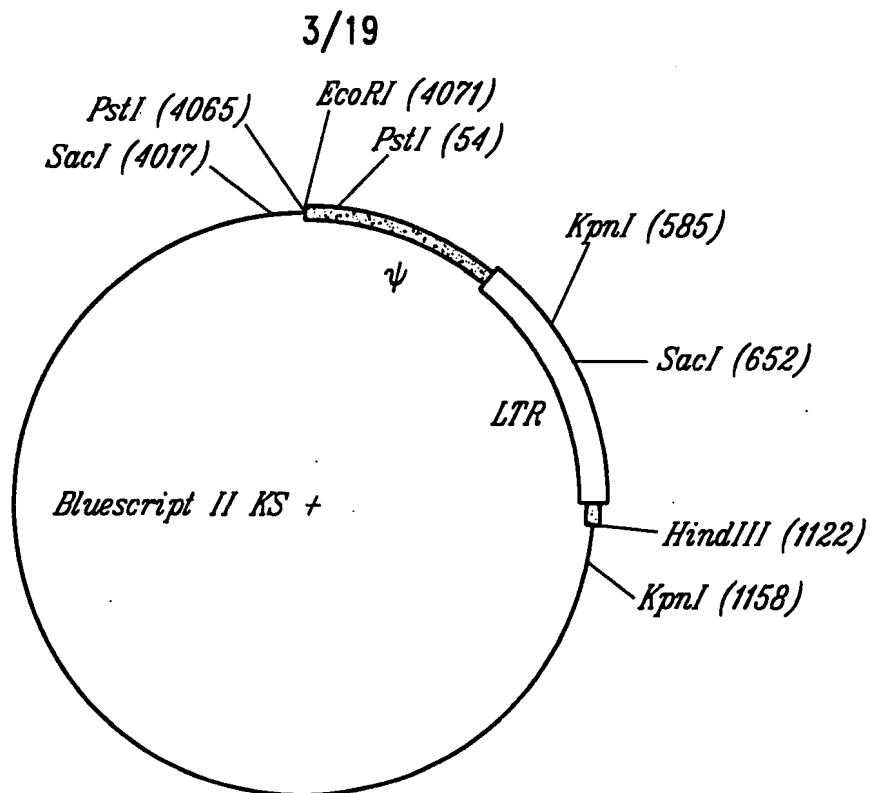


Fig. 3

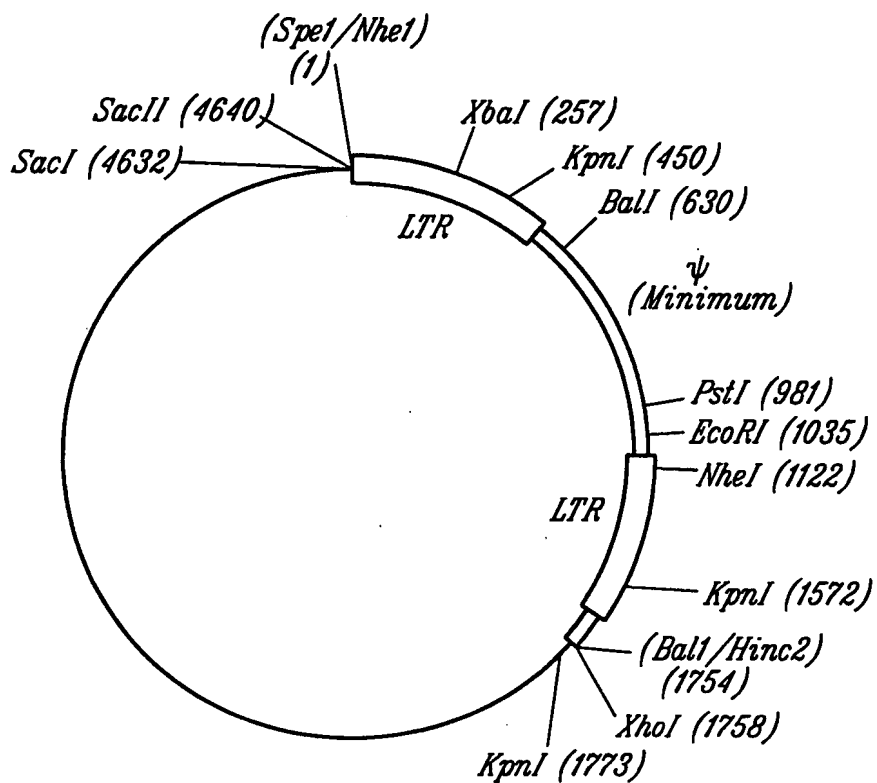
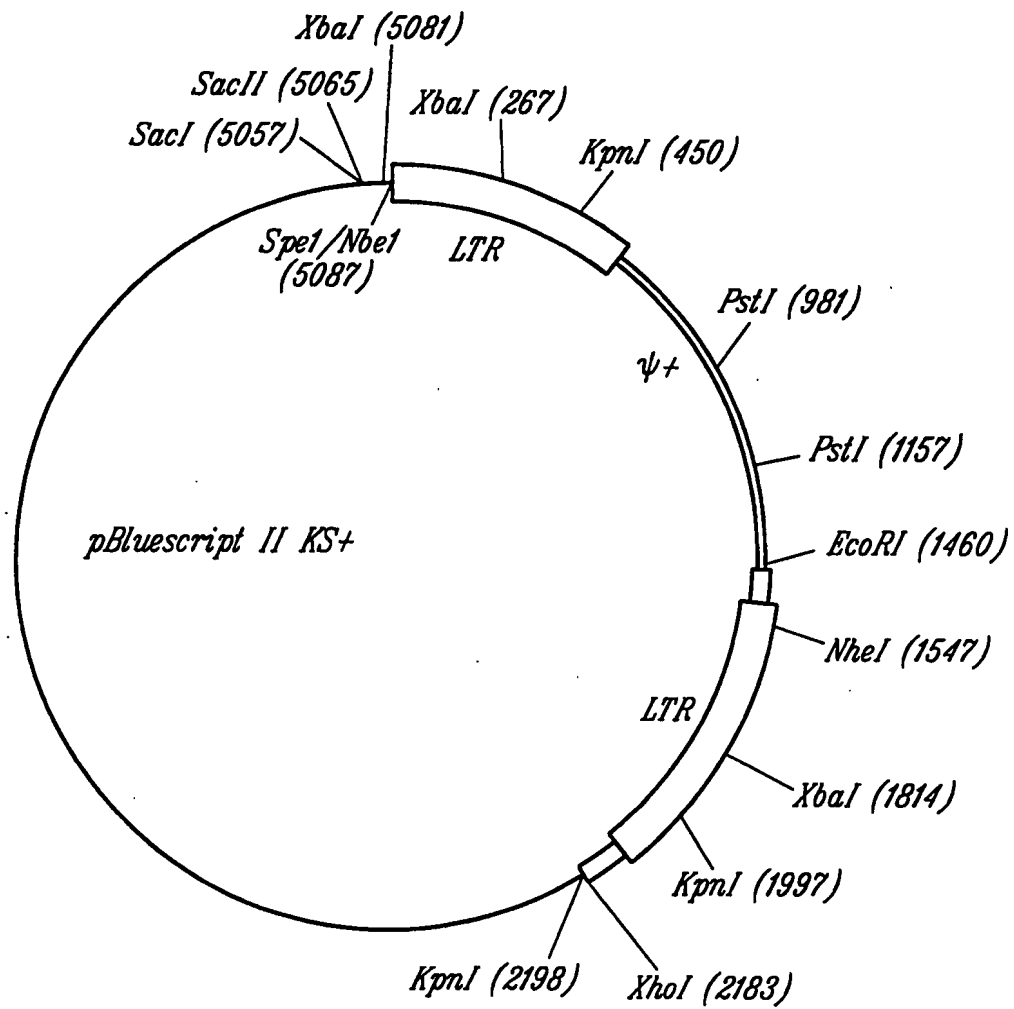


Fig. 4

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*Fig. 5*

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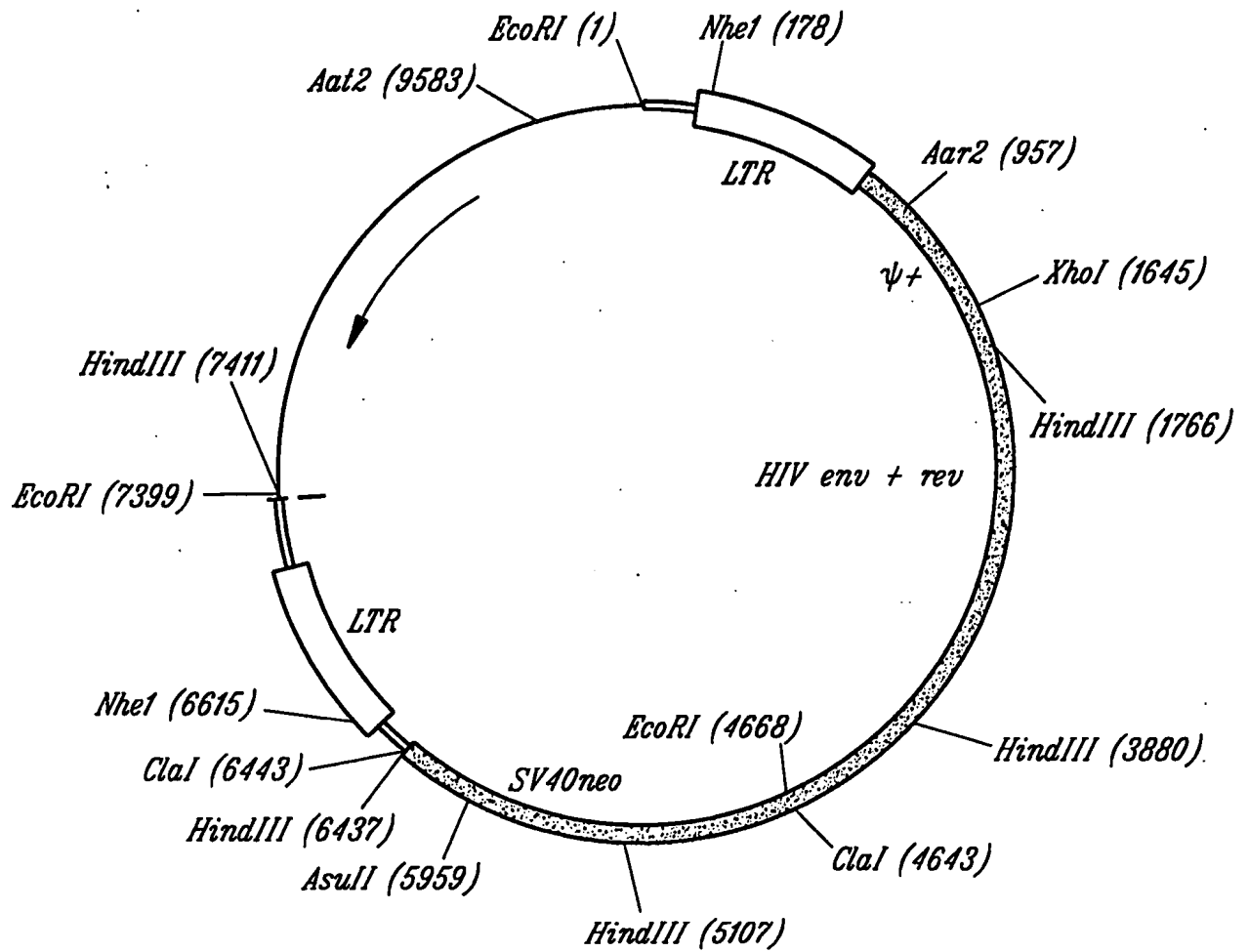


Fig. 6

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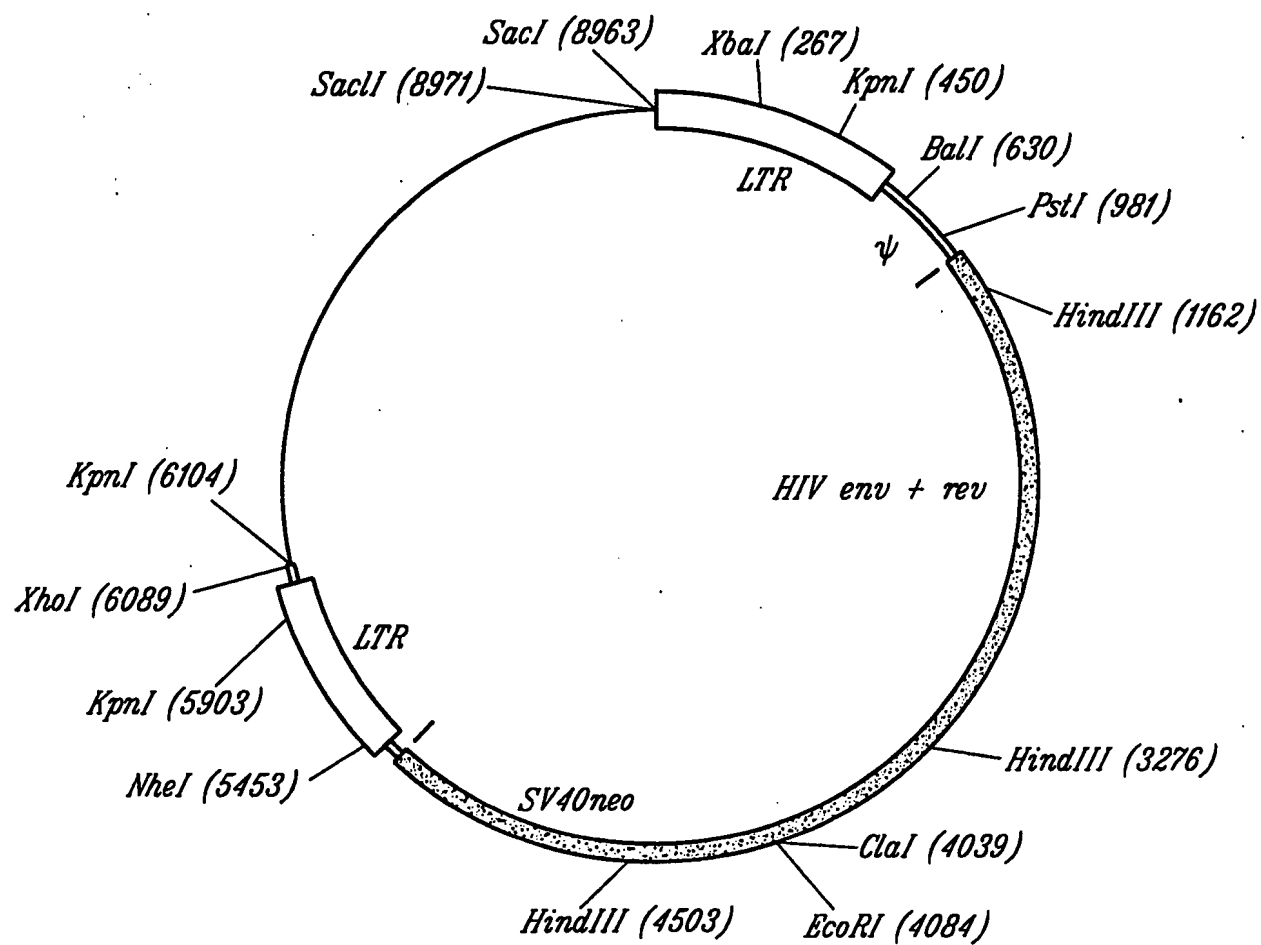


Fig. 7

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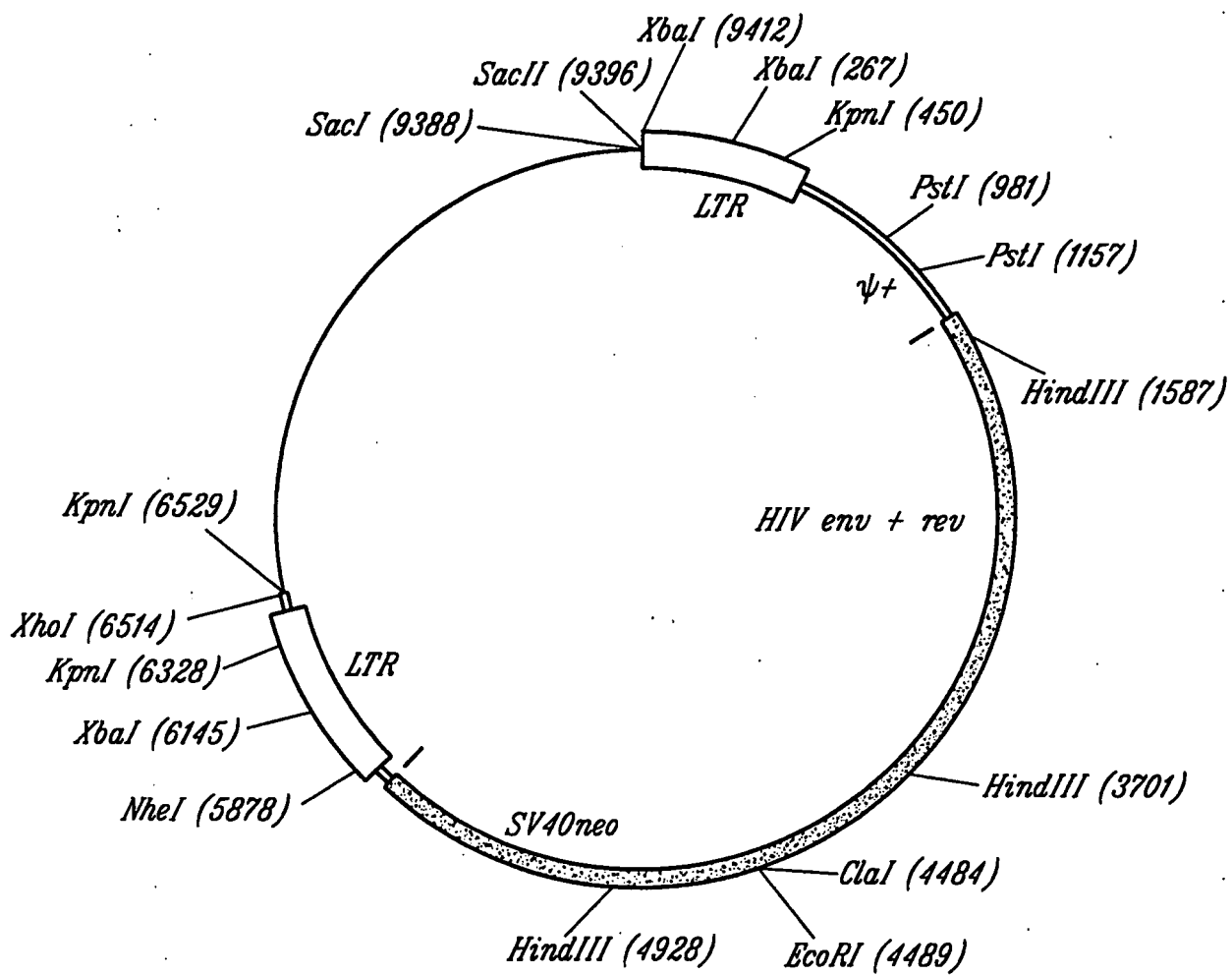


Fig. 8

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1 ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG AAA
 1▶Met Gly Gln Thr Val Thr Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp Lys
 52 GAT GTC GAG CGG ATC GCT CAC AAC CAG CAG TCG CCA ACC TTT AAC GTC GGA TGG CCG
 18▶Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys Arg Arg
 103 TGG GTT ACC TTC Phe Thr TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC GGA TGG CCG
 35▶Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val Gly Trp Pro
 154 CGA GAC GGC ACC TTT AAC CGA GAC GAC CTC ATC ACC CAG GTT AAG ATC AAG GTC
 52▶Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val Lys Ile Lys Val
 205 TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC CCC TAC ATC GTC ACC
 69▶Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val Pro Tyr Ile Val Thr
 256 TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG GTC AAG CCC TTT GTA CAC
 86▶Trp Glu Ala Leu Ala Phe Asp Pro Pro Trp Val Lys Pro Phe Val His
 307 CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC GCC CCG TCT CTC CCC CTT GAA
 103▶Pro Lys Pro Pro Pro Pro Leu Pro Pro Ala Pro Ser Leu Pro Pro Leu Glu
 358 CCT CCT CGT TCG ACC CCG CCT CGA TCC TCC CTT TAT CCA GCC CTC ACT CCT
 120▶Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro
 Narl (415)
 409 TCT CTA GGC GCC
 137▶Ser Leu Gly Ala

Fig. 9

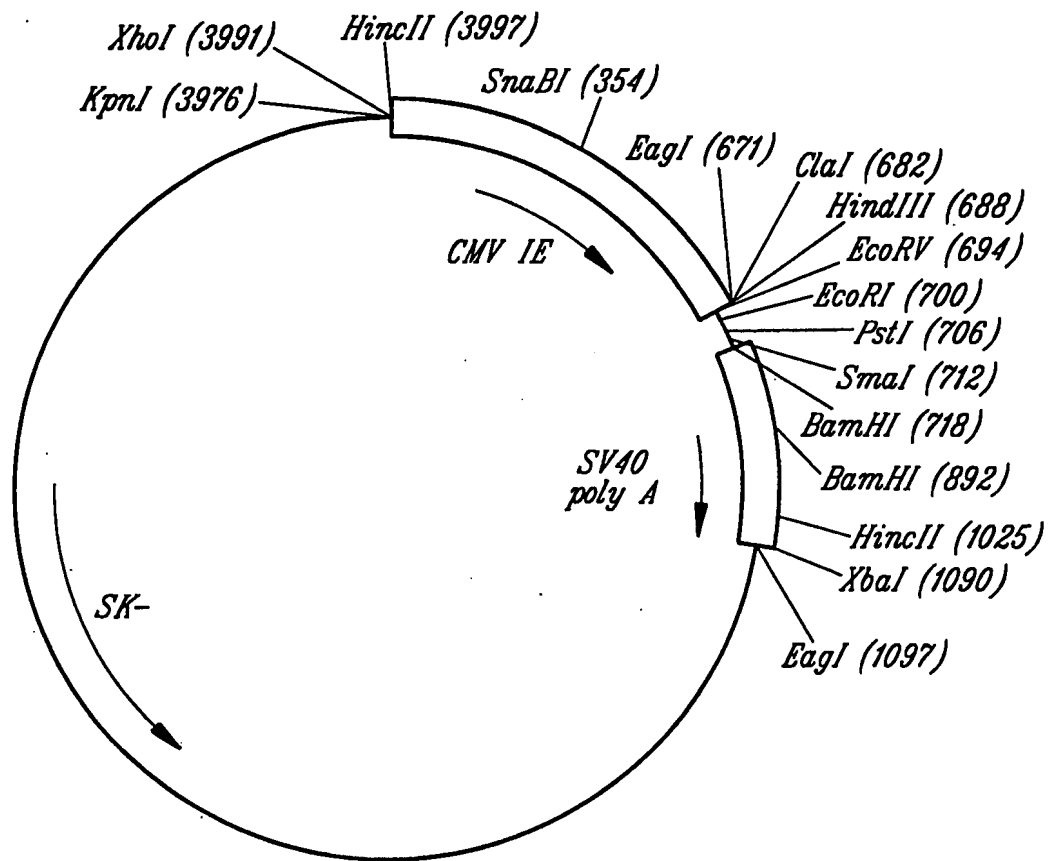
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A TAT ATA TAT ATC GAT ACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
 ▶ Met Gly Gln Thr Val Thr Thr Pro Leu Ser
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 ▶ Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser
 GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
 ▶ Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro
 ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
 ▶ Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile
 ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
 ▶ Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp
 CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
 ▶ Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro
 TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
 ▶ Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
 GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
 ▶ Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

 Narl
 TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA CCT AAA C
 ▶ Leu Tyr Pro Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ???????

Fig. 10

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*Fig. 11*

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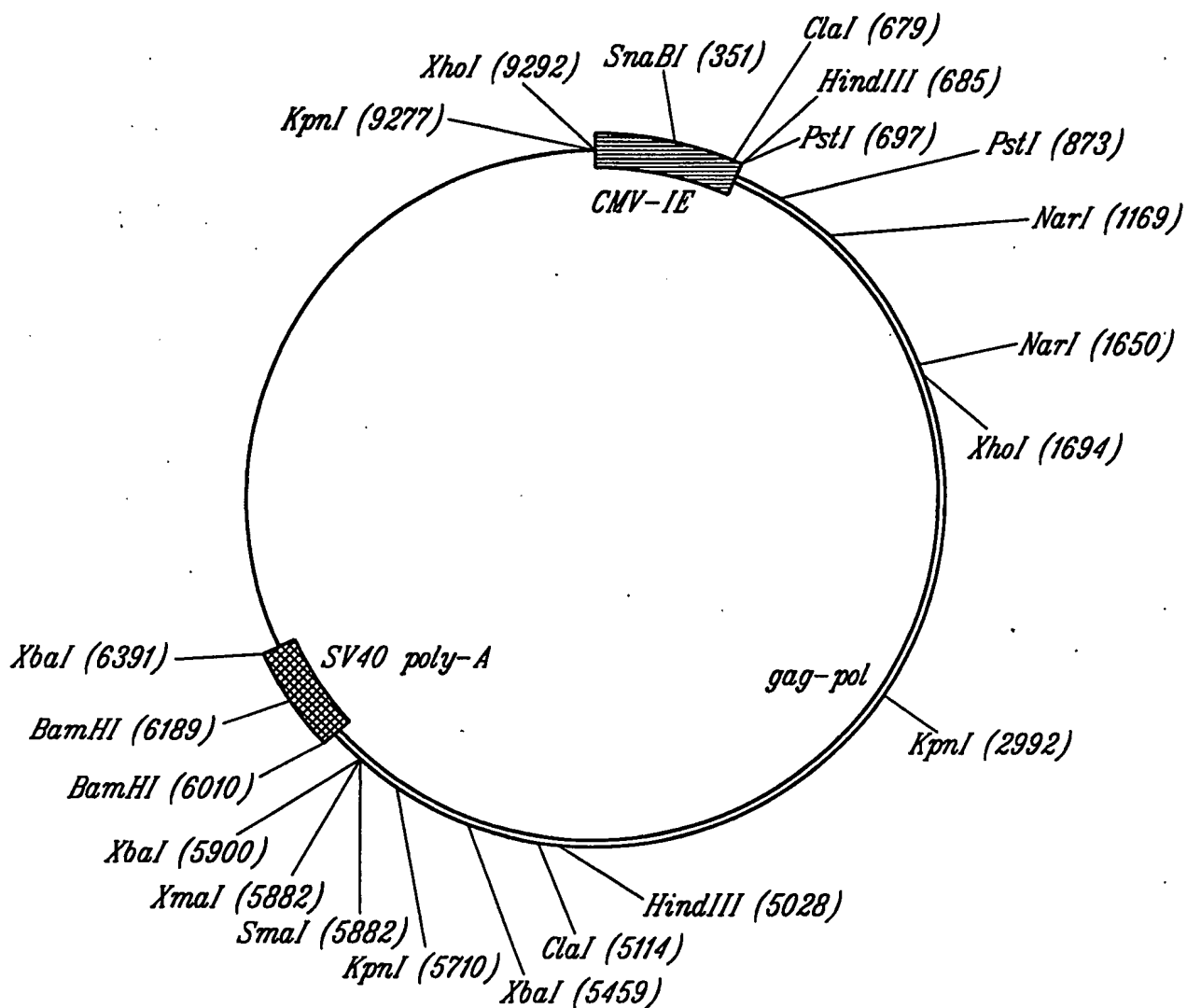


Fig. 12

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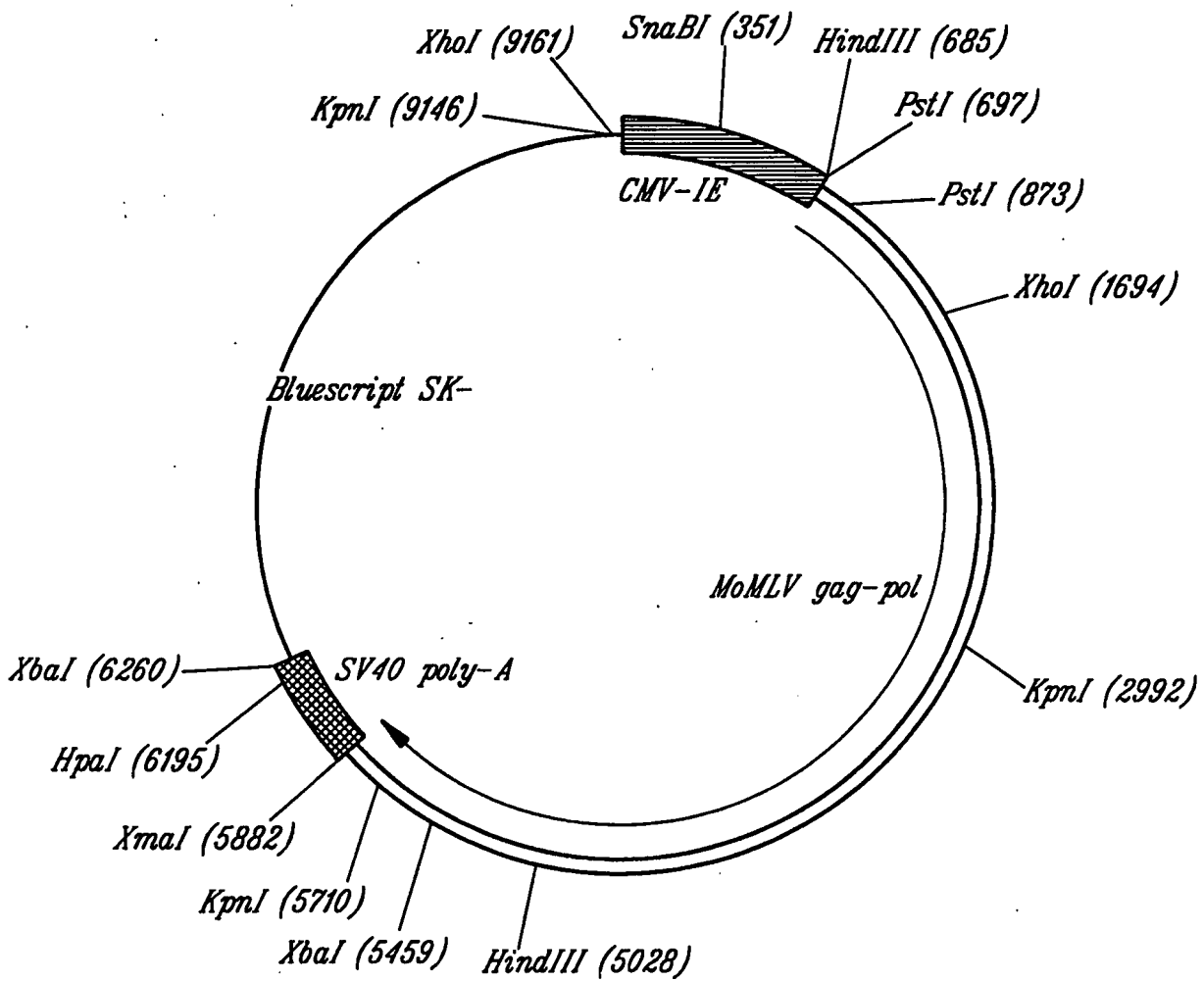


Fig. 13

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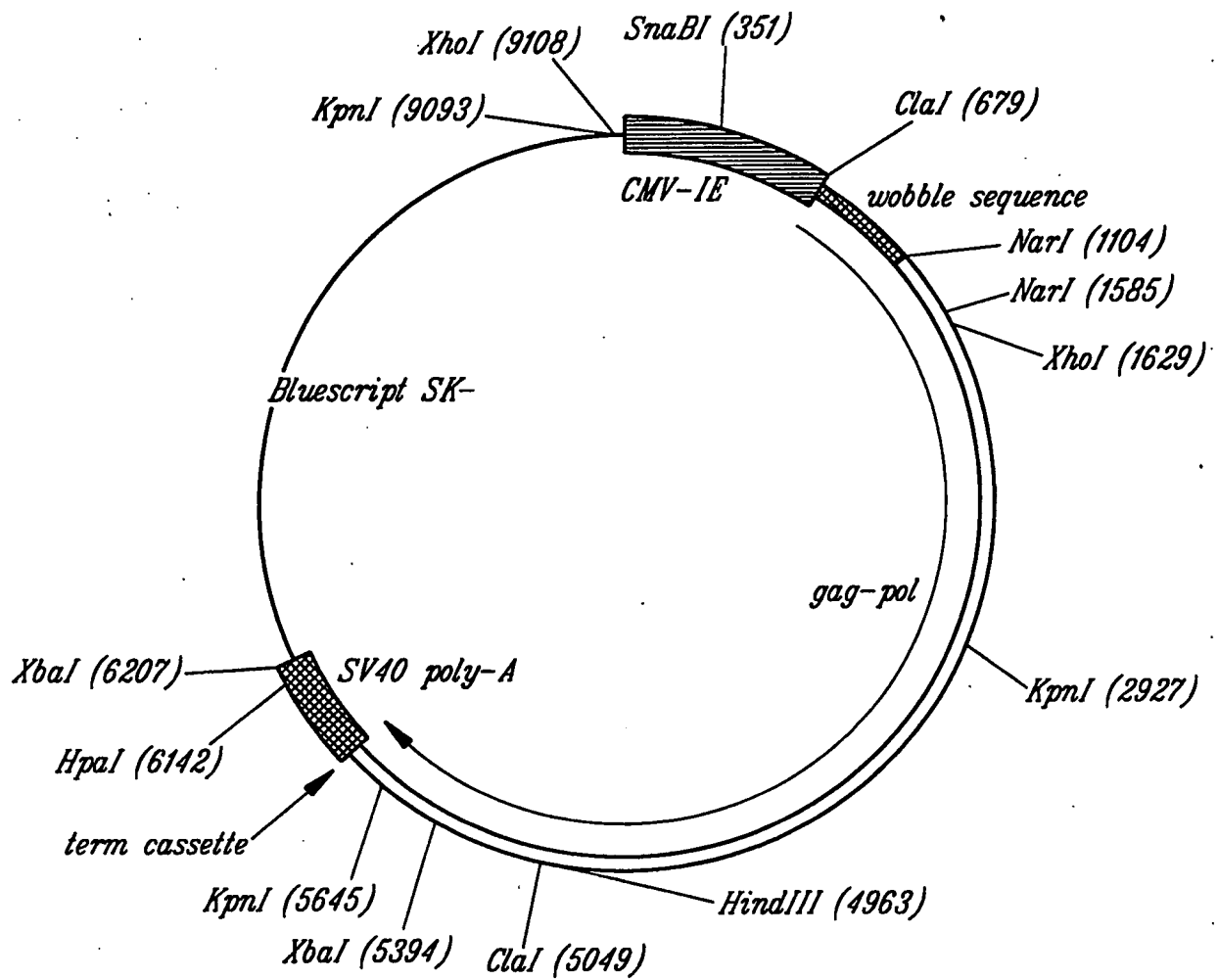


Fig. 14

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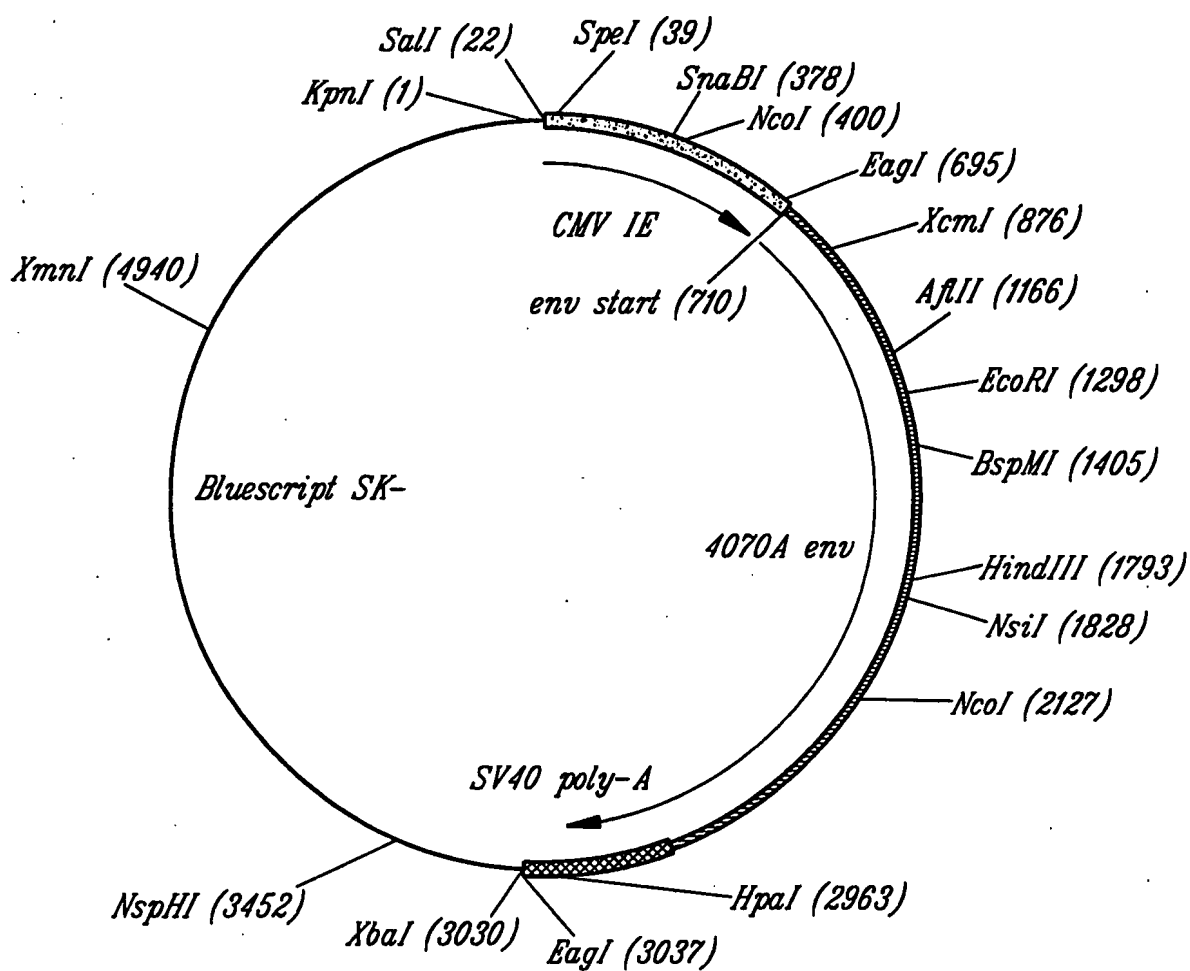


Fig. 15

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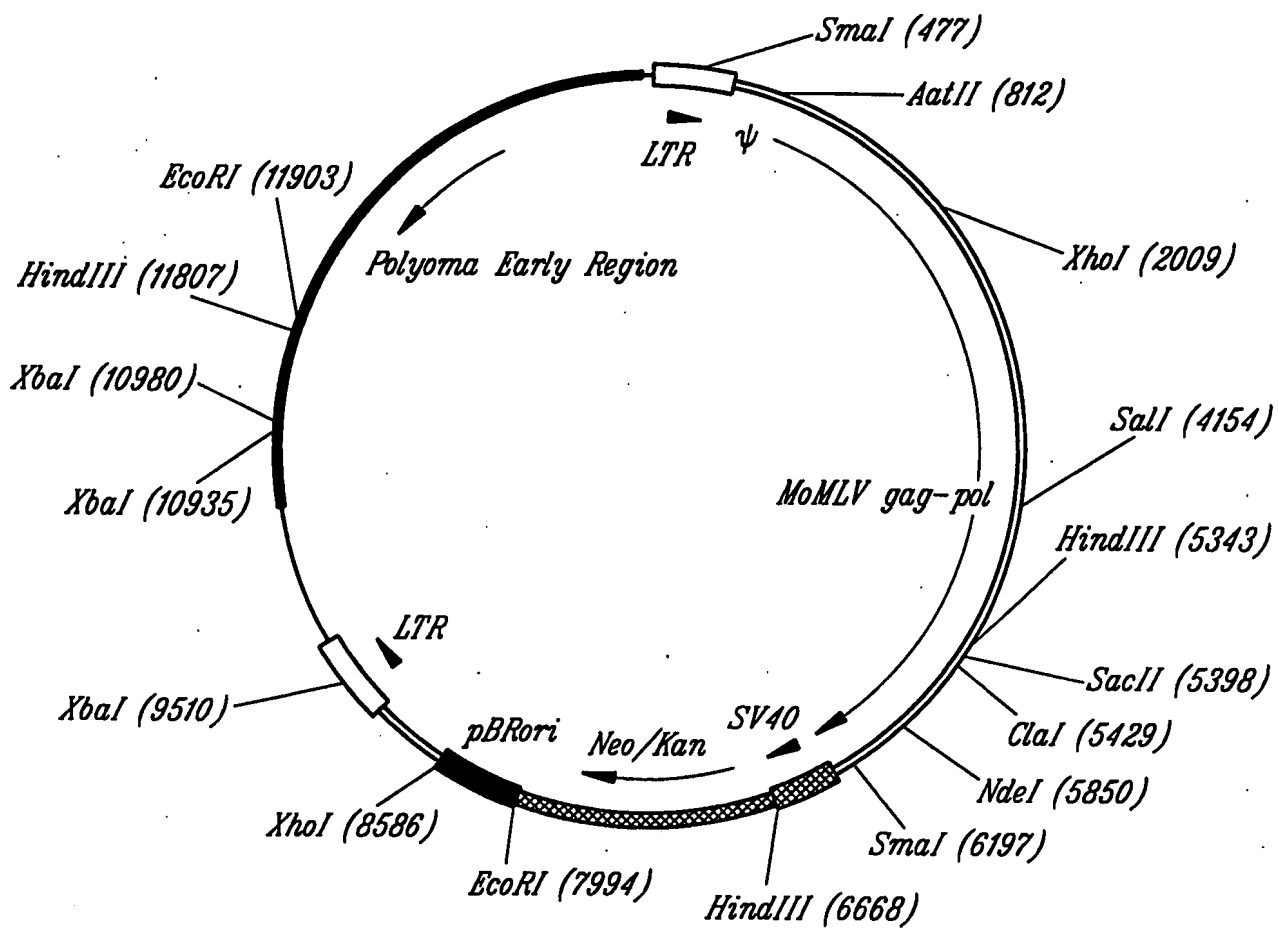


Fig. 16

Fig. 17A

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VIRUS	SPECIES OF ISOLATION	TYPE ¹
AEV (Avian erthroblastosis virus)	chicken	C,X,T
ALV (avian leukosis virus)	chicken	C,N or X,N
AMV (avian myeloblastosis virus)	chicken	C,X,T
ASV (avian sarcoma virus)	chicken	C,X,T
BaEV (baboon endogenous virus)	baboon (<i>Papio ssp.</i>)	C,N,N
B1LN	<i>P. hamadryas</i>	
M7	<i>P. cynocephalus</i>	
M28	<i>P. cynocephalus</i>	
PP-1-Lu	<i>P. papio</i>	
TG-1-K	gelada	
BLV (bovine leukemia virus)	cow	C,X,N
BSV (bovine syncytial virus)	cow	S,X,N
CAEV (caprine arthritis-encephalitis virus)	goat	L,X,N
CERV-CI, CERV C-II	<i>Mus cervicolor</i>	C,N,N
CCC	cat	C,N,N
CPC-1	colobus monkey	C,N,N
CSRV (corn snake retrovirus)	corn snake	C,
CSV (chick syncytial virus)	chicken	C,X,N
DIAV (duck infectious anemia virus)	duck	C,X,N
DKV (deer kidney virus)	black-tailed deer	C,N,N
DPC-1	agouti	C,N,N
EIAV (equine infectious anemia virus)	horse	C,X,N
ESV (Esh sarcoma virus)	chicken	C,X,T
FeLV (feline leukemia virus)	cat	C,N or X,N
FeSV (feline sarcoma virus)	cat	C,X,T
GA (Gardner-Arnstein)		
SM (McDonough)		
ST (Snyder-Theilen)		
FS-1	<i>Felis sylvestris</i> (wildcat)	C,N,N
FSFV (feline syncytium-forming virus)	cat	S,X,N
FuSV (Fujinami sarcoma virus)	chicken	C,X,T
GALV (gibbon ape leukemia virus)	gibbon	C,X,N
GLV (goat leukoencephalitis virus)	see CAEV	
GPV (golden pheasant virus)	golden pheasant	C,N,N
HaLV (hamster leukemia virus)	hamster	C,N,N
IVL (induced leukemia virus)	chicken	C,N,N
LLV (lymphoid leukosis virus)	see ALV	
LPDV (lymphoproliferative disease of turkeys)	turkey	C,X,T
M432	<i>Mus cervicolor</i>	B,N,N
M832	<i>Mus caroli</i>	B,N,N

¹ The first letter denotes classification: (B) B-type oncovirus; (C) C-type oncovirus; (D) D-type oncovirus; (L) lentivirus; (S) spumavirus. The second letter denotes origin: (N) enogenous; (X) exogenous; (R) recombinant. The third letter denotes ability to induce morphological transformation: (T) transforming (i.e., containing an *onc* sequence); (N) nontransforming; (?) unknown.

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MAC-1	stumptail monkey	C,N,N
Maedi	sheep	L,X,N
MAV (myeloblastosis-associated virus)	chicken	C,X,N
MC29 (myelocytomatosis virus)	chicken	C,X,T
MCF (mink cell focus-inducing virus)	mouse	C,NR,N
MH2 (myelocytomatosis virus)	chicken	C,X,T
MiLV (mink leukemia virus)	mink	C,N,N
MLV (murine leukemia virus)	mouse	C,X or N,N
Ab (Abelson)		C,X,T
Fr (Friend)		C,X,N
Graffi		C,X,N
Gross		C,N,N
Ki (Kirsten)		C,X,N
Mo (Moloney)		C,X,N
Ra (Rauscher)		C,X,N
MMC-1	rhesus monkey	C,N,N
MMTV (mouse mammary tumor virus)	mouse	B,X or N,N
MPMV (Mason-Pfizer monkey virus)	rhesus monkey	D,X,N
MSV (murine sarcoma virus)	mouse	C,X,T
BALB		
FBJ (Finkel-Biskis-Jinkins)		
FBR		
Gz (Gazdar)		
Ha (Harvey)		
Ki (Kirsten)		
Mo (Moloney)		
MPV ¹ (myeloproliferative)		
OS2 (osteosarcoma)		
MyLV (myeloid leukemia)	mouse	C,X,N
OK10 (myelocytomatosis virus)	chicken	C,X,T
OMC-1	owl monkey	C,N,N
PK-15	pig	C,N,N
PO-1-Lu	langur	D,N,N
PPV (progressive pneumonia virus)	sheep	L,X,N
PRCII, PRCIV (Poultry Research Centre)	chicken	C,X,T
R-35	rat	C,X?,T
RaLV (rat leukemia virus)	rat	C,X,N
RaSV (rat sarcoma virus)	rat	C,X,T
RAV- <i>n</i> (Rous-associated virus)	see ALV	
RAV-0 (Rous-associated virus 0)	chicken	C,N,N
RAV-60 (Rous-associated virus 60)	chicken	C,R,N
RAV-61 (Rous-associated virus 61)	ring-necked pheasant	C,R,N
RD114	cat	C,N,N
REAV (reticuloendotheliosis-associated virus)	turkey	C,X,N

Fig. 17B

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REV (reticuloendotheliosis virus)	birds	C,X,N
REV-T (reticuloendotheliosis virus-transforming)	turkey	C,X,T
RIF (Rous interference factor)	see ALV	
RPL- <i>n</i> (Regional Poultry Laboratory)	see ALV	
RPV (ring-necked pheasant virus)	ring-necked pheasant	C,R,N
RSV (Rous sarcoma virus)	chicken	C,X,T
B77 (Bratislava)		
BH (Bryan high titer)		
BS (Bryan standard)		
CZ (Carr-Zilber)		
EH (Engelbreth-Holm)		
HA (Harris)		
PR (Prague)		
SR (Schmidt-Ruppin)		
SFV- <i>n</i> (simian foamy virus)	monkey	S,X,N
SFFV (spleen focus-forming virus)	mouse	C,X, or R,N or T
Friend		
MPV		
Rauscher		
SiSV (simian sarcoma virus)	see SSV	
SLV (simian lymphoma virus)	see GALV	
SMRV (squirrel monkey retrovirus)	squirrel monkey	D,N,N
SMV (simian myelogenous leukemia virus)	see GALV	
SSAV (simian sarcoma-associated virus)	woolly monkey	C,X,N
SSV (simian sarcoma virus)	woolly monkey	C,X,T
TRV-1	tree shrew	C,N,N
UR- <i>n</i> (University of Rochester)	chicken	C,X,T
Vand C-I	tree mouse	C,N,N
Visna	sheep	L,X,N
VRV (viper retrovirus)	Russell's viper	C,N,?
WMV (woolly monkey virus)	see SSV	
WoLV (woolly monkey leukemia virus)	see SSAV	
Y73 (Yamaguchi 73)	chicken	C,X,T

Fig. 17C

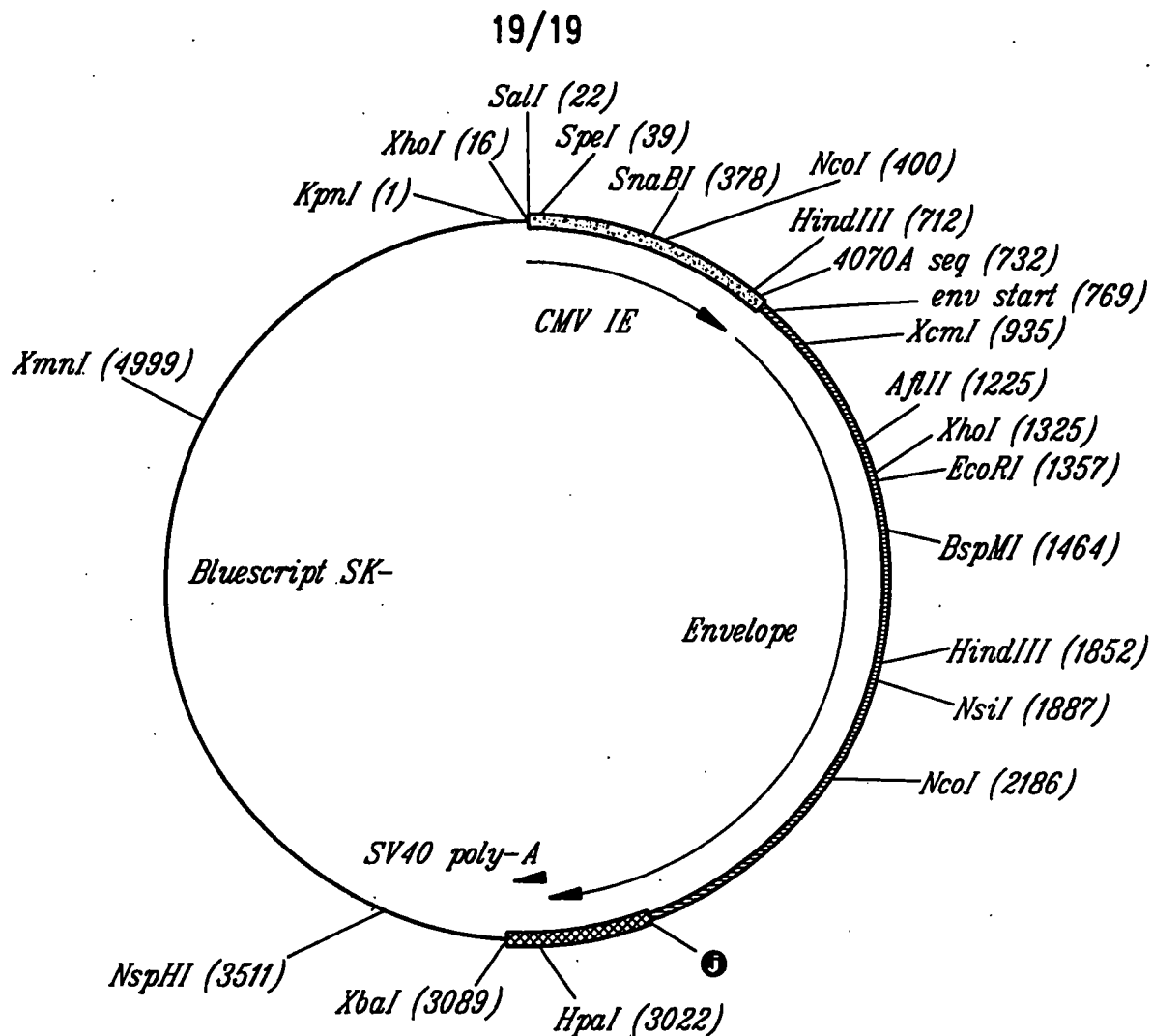


Fig. 18

CMV Promoter — wobble gag — SVneo — LTR

Fig. 19A

CMV Promoter — normal gag — SVneo — LTR

Fig. 19B



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 5/10, 7/04, 9/00, C07K 14/00, 14/005, 14/15, 14/54	A3	(11) International Publication Number: WO 95/30763 (43) International Publication Date: 16 November 1995 (16.11.95)
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(21) International Application Number: PCT/US95/05789

(22) International Filing Date: 9 May 1995 (09.05.95)

(30) Priority Data:
08/240,030 9 May 1994 (09.05.94) US(71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street,
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Diego, CA 92109 (US).(74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300
Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-
7092 (US).(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ,
EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK,
LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO,
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patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE,
MW, SD, SZ, UG).**Published***With international search report.**Before the expiration of the time limit for amending the
claims and to be republished in the event of the receipt of
amendments.*(88) Date of publication of the international search report:
4 April 1996 (04.04.1996)

(54) Title: RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

A TAT ATA TAT ATC GAT ACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
 ▶ Met Gly Gln Thr Val Thr Thr Pro Leu Ser

CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
 ▶ Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
 ▶ Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
 ▶ Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
 ▶ Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
 ▶ Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
 ▶ Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
 ▶ Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

Next

TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA CCT AAA C
 ▶ Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????

(57) Abstract

Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral *gag/pol* or *env* coding sequences. In addition, *gag/pol*, and *env* expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, *gag/pol* and *env* expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 C12N7/04 C12N9/00 C07K14/00
C07K14/005 C07K14/15 C07K14/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.VIROLOGY, vol. 66, no. 3, March 1992 page 1571-1578 T. TCHENIO AND T. HEIDMANN 'High-frequency intracellular transposition of a defective mammalian provirus detected by an In Situ colorimetric assay' see the whole document ---	1,6-8
P,X	WO,A,94 29438 (CELL GENESYS, INC.) 22 December 1994 see page 5, line 11 - page 7, line 35 see page 9, line 5 - page 12, line 32 see page 14, line 16 - page 21, line 22 --- -/--	22-26, 28-37, 39-46, 48-51

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Date of the actual completion of the international search

29 January 1996

Date of mailing of the international search report

29.02.96

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J.VIROLOGY, vol. 68, no. 6, June 1994 pages 3888-3895, C. PAROLIN ET AL. 'Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes' see the whole document ----	1,6
Y	WO,A,89 07150 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 10 August 1989 see the whole document ----	1-52
Y	VIROLOGY, vol. 167, 1988 pages 400-406, D. MARKOWITZ ET AL. 'Construction and use of a safe and efficient amphotropic packaging cell line' see the whole document ----	1-52
Y	PROC.NATL.ACAD.SCI. USA, vol. 85, 1988 pages 6460-6464, O. DANOS AND R.C. MULLIGAN 'Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges' see the whole document ----	1-52
Y	WO,A,90 02806 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 22 March 1990 see the whole document ----	1-52
Y	INT.CONF.AIDS (NETHERLANDS), JULY 19-24 1992, vol. 8, no. 2, 1992 page PA82, Abstract No. POA 2477 V. NEUBAUER ET AL. 'Construction of a retroviral vector for intracellular immunization with a HIV antisense sequence' see the whole document ----	11
A	PROC.NATL.ACAD.SCI. USA, vol. 84, 1987 pages 156-160, J. PRICE ET AL. 'Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer' see the whole document -----	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9429438	22-12-94	AU-B- 7246294	03-01-95
WO-A-8907150	10-08-89	AU-B- 3069189	25-08-89
		CA-A- 1315719	06-04-93
		US-A- 5278056	11-01-94
WO-A-9002806	22-03-90	EP-A- 0432216	19-06-91
		JP-T- 4500158	16-01-92
		US-A- 5449614	12-09-95